Bioprospecting Surfactants Produced by *Pseudomonas* spp. Isolated from Soil for Potential Application in Biotechnology

A thesis submitted for the degree of Doctor of Philosophy (PhD)

by

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April, 2017

Certification

I, Andrew Spiers, hereby certify that the candidate has fulfilled the conditions of the Resolutions and Regulations appropriate for the degree of Doctor of Philosophy (PhD) at Abertay University, and that the candidate is qualified to submit this thesis in application for that degree.

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Declarations

I, **Kamaluddeen Kabir**, hereby certify that this thesis submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy (PhD), Abertay University, is wholly my own work unless otherwise referenced or acknowledged. This work has not been submitted for any other qualification at any other academic institution.

02/08/17

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Acknowledgements

I would like to express my sincere gratitude to my supervisor, Dr Andrew Spiers. He has continuously supported me during my PhD programme at Abertay University, and his patience, motivation and advice on ideas have been a source of inspiration to me. This thesis would not have been accomplished without his intellectual input. His supervision has guided me throughout my research and writing of this thesis, and I cannot imagine having a better supervisor or mentor.

My immense gratitude also goes to my second supervisor, Dr Yusuf Deeni, for his intuitive comments and support. His questions and input truly helped me to widen my research and look at it from different angles. Even with his tight schedule, he took the time to go through my entire thesis and for this I have nothing to say but, 'JazakAllah khair'. My sincere gratitude also goes to Zuzana Spiers for her help during the Sample collection and for making my stay in Dundee a memorable one. I also hereby extend my gratitude to the Head of Graduate School, Dr Nia White and her assistance Miss Wendy Robb for their support and guidance.

I remain indebted to my parents Alhaji Kabir and Hajiya Halima for their extensive role in my upbringing and education. Their continuous support in any times of difficulty cannot be under-estimated: they will forever be precious in my heart. My gratitude goes to my step mother, Hajiya Sa'adatu and to my brothers and sisters Khadija, Zaharaddeen, Nafisa, Nura, Aisha, Saratu and Pharm. Muhammad, and to Hayatu, Sadam, Binta, Farida, Jalaluddeen, Lubabatu, Tajuddeen, Ahmed and Kamaraddeen, as well as to all those I have not mentioned but who are not forgotten. To my uncles, aunts, nephews and nieces, thank you for all your prayers and concern.

To my wife Aisha and daughter Saratu, thank you for your continuous understanding and motivation. You gave me joy when I was tired and kept me on track - thank you. And to my in-laws Muhammad Jamil Yusuf and family. A final thanks also goes to Uncle Ismail and family, thank you for your kindness, care and support in making life easier in Dundee.

I would like to thank my colleagues at the Graduate School for their interesting discussions and all the fun we have had over the last few years. In particular, I would like to acknowledge Ibrahim Kankia and Raji.

A big thanks is owed to my friends here in the United Kingdom including Solomon Anjide, Musa Ladan, Aliyu and Armaya'u. My conscience would not rest if I did not acknowledge my friends back home, Abdullateef, Nura, Umar Zango, Auwal Dansharif, Abubakar, Jamil Bashir, Baba Bala, Aminu Dankanjiba, Ibrahim Barguy, and others too many to name for their spiritual and verbal support throughout my stay at Abertay.

A special thanks goes to Prof. Mu'utasim Ibrahim and family and to Engr. Muttaqha Rabe Darma for their part in supporting me in attaining this level of my career; Allah will surely reward you. I also hereby extend my gratitude to Malik Anas, Dr Dikko Suleiman and Family, Alhaji Galadima and Hajiya Jummai of PTDF and Muhammad Ahmad and Hayatu Ruma of Umaru Musa Yar'adua University.

Last but not least, I would like to acknowledge my sponsors Umaru Musa Yar'adua University Katsina (UMYU) and the Tertiary Education Trust Fund (TETFUND) for sponsoring my research at Abertay University, Dundee, United Kingdom. My thanks also to the other societies that sponsored some of my conference attendance.

Dedication

This thesis is dedicated to my beloved parents, Alhaji Kabir Mohammed and Hajiya Halima Ali Yar'adua, for all their help, caring and encouragement, and for raising me and giving me hope in life. May Allah continue to guide and protect them in all their doings. And to my wife Aisha and daughter Saratu Kamaluddeen, I dedicate this to you both for being my source of happiness.

Abstract

Bacteria produce a range of surface-active compounds called biosurfactants that reduce the surface tension of liquid and exhibit different oil-water behaviours. These are used in various biotechnological applications including agriculture, cosmetics, medical and food. A recent study has predicted a limit to bacterial surface tension-reducing ability. If this limit exists, it has strong negative consequences in surveys for more active compounds. In this work, the aim is to (i) investigate this prediction more robustly by using chemical media and (ii) study the diversity amongst the best-performing surfactants produced by *Pseudomonas* spp. with the intention of finding novel surfactants that could be used in different biotechnological applications.

A total of 251 *Pseudomonas* spp. were isolated from soil. Strains were first screened for liquid surface tension-reducing ability (LSTRA) using qualitative drop-collapse assay before quantitative surface tension measurement. Of the 58 LSTRA strains, only 46 significantly reduced the surface tension of sterile media. Individual Distribution Identification (IDI) analysis was used to determine the predicted limit for surfactant activity in KB* and M9Glu media, and results were found to be in agreement with earlier studies.

To investigate the chemical structural diversity amongst the best performing surfactants, a collection of 25 key strains producing a limited range of very low surface tension in liquid culture media (~24 – 26 mN/m) were examined. Initial phenotypic characterisation including biochemical, metabolic profiling and 16S rDNA sequencing confirmed strains were a diverse collection of *Pseudomonas* spp.. A series of behaviour assays including emulsion formation, foam stabilisation and oil displacement assays to investigate behavioural diversity among surfactants expressed by the key strains were then undertaken. For the oil displacement, diesel, mineral, vegetable, and used lubricating oils were tested with the underlying aqueous layer containing 0 or 200 mM NaCl at pH 6.0 or 8.0 to reflect a range of biotechnological applications and conditions. Analysis of variance of the emulsion indices, foam stabilisation and oil displacement data showed significant difference in surfactant behaviour among the key surfactant-expressing strains ($P < 0.001$). Moreover, Hierarchical Cluster Analysis (HCA) was used to produce a constellation dendrogram in which isolates were grouped according to similarities in phenotype and surfactant behaviour. Critically, this resulted in more groups (≥ 5 groups) than could be explained by statistically significant differences in mean surface tensions (previously determined by ANOVA and Tukey-Kramer HSD, *alpha* = 0.05). These findings provide strong evidence that the key strains were expressing structurally more than one type of surfactant with differing air-water and oil-water behaviours. Similarly, *in vitro* surfactant characterisation within a range of pH and salt concentrations confirmed diversity among strains ($P < 0.001$). Investigating surfactant potential by a two-way behaviour cluster dendrogram resulted in more diversity among oil types than the conditions used. These findings indicate that bioprospecting surfactants by screening only the more active compounds is likely to reveal a range of functionalities.

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Definitions

AD – Anderson-Darling

ANOVA – Analysis of Variance

API – Analytical Profilling Index

CLP – Cylic Lipopeptide

GLM – Generalised Linear Model

HCA – Hierarchical Cluster Analysis

IDI – Individual Distribution Identification analysis

LSTRA – Liquid Surface Tension Reducing Ability

SAC – Surface Active Compounds

SE – Standard Error

TK-HSD – Tukey Kramer Honest Significant Difference

ULO – Used lubricating oil

Chapter 1

Introduction

Preface

Biosurfactants that reduce the surface tension of liquid the most are receiving researchers' attention worldwide because of their potential in biotechnology. In order to prospect biosurfactants for different applications in biotechnology, a large collection of bacterial strains were isolated, with a particular interest in pseudomonads, and were screened for biosurfactant expression using a high-throughput technique. These were later subjected to quantitative surface tension measurement. Surfactants that reduced the surface tension the most were subsequently studied using a range of behavioural assays to establish chemical structural diversity and to test them for varied applications in biotechnology. The aim of this Chapter is to introduce literature directly related to the research objectives of this thesis. Biosurfactants and their potential applications, isolation and purification of surfactants, surfactant activity and the minimum limit and surfactant chemical structural diversity are discussed. This is followed by the study rationale of this thesis and introduction of its main research objectives.

Chapter 1 Introduction

1.1 An introduction to Biosurfactants and their application

Biosurfactants are surface-active compounds produced by bacteria. They contain both hydrophilic (that interact with water) and hydrophobic (that do not interact with water) chains within their structures which can accumulate and reduce water surface tension in air- or oil-water interfaces (reviewed by Banat, 1995, Raaijmakers *et al.*, 2006)**.** Biosurfactants that reduce the surface tension of water the most are increasingly finding importance in biotechnology (Beal and Betts, 2000, Christofi and Ivshina, 2002, Singh *et al.*, 2007). They are similar to chemical surfactants in behaviour and function but unlike chemical surfactants, they are less toxic, biodegradable or biocompatible (Benvegnu *et al.*, 2008, reviewed by Mondal *et al.*, 2015, Mudgil, 2011). Recently, great attention has been given to the environmental impact caused by chemical surfactants due to their high toxicity and related environmental health concerns. Rapid developments in biotechnology have led to biosurfactants being a potential alternative to chemical surfactants (reviewed by Banat *et al.*, 2000, Henkel *et al.*, 2012, Reis *et al.*, 2013, Van Hamme *et al.*, 2006).

Biosurfactants have a natural role in phosphate solubilisation, antibacterial activity, phytohormone production, biocontrol, swarming motility, quorum sensing, biofilm formation and induced systematic resistance in plants (reviewed by Raaijmakers *et al.*, 2006, Raaijmakers *et al.*, 2010, Ron and Rosenberg, 2001, Rosenberg and Kjelleberg, 1986). Furthermore, biosurfactants are useful in different applications of

biotechnology such as agriculture, food, medical, pharmaceutical and petroleum industries (where they are used to enhance oil recovery and bioremediation) (Brown *et al.*, 1985, Burt, 2004, Gudiña *et al.*, 2013, Inès and Dhouha, 2016, Islam *et al.*, 2015, Kalia and Mudhar, 2011, Sachdev and Cameotra, 2013, Salim *et al.*, 2014, Salwiczek *et al.*, 2014). For the purpose of this thesis, biosurfactants will be referred to as surfactants.

1.2. Isolation and purification of surfactants

Isolation of surfactant producing organisms normally begins with a survey of a large collection of bacterial isolates that are screened using qualitative assays to select surfactant-expressing strains. Simple qualitative assays such as the drop-collapse technique, blood haemolysis agar and oil-water behaviour have been used to do this (Bharali *et al.*, 2011, Bodour and Miller-Maier, 1998, Carrillo *et al.*, 1996, El-Sheshtawy and Doheim, 2014, Thavasi *et al.*, 2008, Vanavil and Rao, 2013). Microbial isolates that are found to be positive are then characterised further, often by assessing the culture supernatants or semi-purified surfactants so as to determine the chemical structure of the purified compounds (Ahmad *et al.*, 2016, Benincasa *et al.*, 2004, reviewed by Hamley, 2015). Surfactant can be partially purified using concentrated acid (de Bruijn *et al.*, 2007, De Souza *et al.*, 2003), while ultra-purification is achieved mostly by using thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) techniques (Kim *et al.*, 2004, Sim *et al.*, 1997).

In another approach, surfactant can be identified by measuring surfactant strength using quantitative tensiometry measuring the liquid surface tension reducing ability (LSTRA) of surfactants. In general, surfactants that lower the surface tension of water the most are generally regarded as strong surfactants with a high surface activity with the bacteria producing these showing significant liquid surface tension-reducing abilities (LSTRA) (Carrillo *et al.*, 1996, Fechtner *et al.*, 2011, Mohammed *et al.*, 2015). Although there is no minimum threshold to which surface active compounds can be identified as surfactant, many studies suggest 22 – 40 Mn/m being reasonable (Mulligan, 2005). Consequently, to identify surfactant with potential conditions for exploitation in biotechnology, significant LSTRA strains are generally selected for further testing (Mulligan, 2005, reviewed by Mulligan *et al.*, 2001, Urum and Pekdemir, 2004).

In addition to the qualitative and quantitative analyses that are useful in high-throughput screening of larger collections, several analytical chemical techniques, including HPLC, MS, thin-layer chromatography and Nuclear Magnetic Resonance (NMR), MS-MS , are used to characterise surfactant chemical structure (Heyd *et al.*, 2008, Miller *et al.*, 2005, Müller *et al.*, 2012, Raaijmakers *et al.*, 2010, Satpute *et al.*, 2010). Other techniques such as the molecular biology approaches that involve the use of whole-genome sequences to identify surfactant-expressing genes, using homology-based techniques and predicts chemical structure and function, are also employed (Hartwell *et al.*, 1999, Marcotte *et al.*, 1999). These techniques are expensive and potentially impossible to apply on a large sample size, even though

they do have sufficient high resolution power to identify and characterise surfactants (Heyd *et al.*, 2008, reviewed by Rahman and Gakpe, 2008, Smyth *et al.*, 2010).

1.3 Surfactant activity and prediction of minimum limit

Surfactant activity has been assessed using a number of techniques including capillary, stalagmometer and tensiometer methods (Bodour and Miller-Maier, 1998, Morikawa *et al.*, 2000), The first bacterial surfactant to be characterised, surfactin produced by *Bacillus subtilis,* could reduce the surface tension of water from 72 mN/m to 27 mN/m (Peypoux *et al.*, 1994). Since then, considerably higher active compounds expressing lower surface-activity have not been reported, despite a large collection of surveys reported (Amani *et al.*, 2013, Antunes *et al.*, 2009, Costa *et al.*, 2006, Gao *et al.*, 2016, Joshi *et al.*, 2015, Morikawa *et al.*, 1993, Moura de Luna, 2010, Nitschke and Pastore, 2006, Rocha e Silva,Nathália Maria P. *et al.*, 2014, Saimmai *et al.*, 2012, Youssef *et al.*, 2004). It is not clear if significantly higher active compounds are being found in new surveys, notwithstanding the limit of 29 mN/m suggested in 2012 based on a 1997 article listing 46 papers (Marchant and Banat, 2012).

Surfactant activity can also be examined using statistical tools to analyse probability distribution model that provides the best fit to the observed data (D'Agostino, 1986). Based on this approach, Fetchner *et al.* (2011) used individual distribution identification (IDI) analysis to study the liquid surface tension-reducing ability of 72

Pseudomonas spp. isolated from sandy loam soil, and predicted a minimum limit of surfactant activity of 24 mN/m. Similarly, Mohammed *et al.* (2015) used IDI analysis to analyse an independent set of pseudomonads isolated from soil and activated sludge and a parallel set of 59 published bacterial LSTRA measurements, and confirmed the limit to be 24 mN/m. However, limits to surfactant strength is suggesting an expression of stronger surfactants is not likely, and thus additional surveys for surfactants with high activity may be less rewarding. Although Mohammed *et al.* (2015) showed evidence of behavioural diversity among surfactants with strong activity that may be of interest to biotechnology (Mohammed *et al.*, 2015, Rosenberg and Kjelleberg, 1986),

The limit of the bacterial surfactant activity lacks detailed explanation. It was thought to result from producing cells' prevention of self-injury; bearing in mind the negative effect surfactants synthesis has on bacterial cells (reviewed by Hatha *et al.*, 2007). Indeed, fatty acid-containing surfactants of amphiphilic nature as a non-polar chain increase their attractiveness to other amphipathic molecules (i.e. biological membranes' lipid components) through non-polar interactions, thereby showing toxicity to bacterial cells (Louvado *et al.*, 2010, Sun *et al.*, 2008). Further studies are needed to ensure that growth media components do not attribute to the theoretical activity limit experience, nor that they are attributable to surfactants and other chemicals presenting biochemical systems interacting in the media, thereby making them unsuitable for stronger surfactant expression.

1.4 Diversity in surfactant behaviour

Surfactant diversity is mostly described in terms of their behaviour and chemical structural composition. Surfactant chemical structural analysis has classified surfactants into four major classes based on hydrophilic and hydrophobic moieties: glycolipids, lipopetides/lipoamino acids, polymers including proteins and polysaccharides, and oil/membranes including fatty acids (Benincasa *et al.*, 2004, Kosaric and Sukan, 1993, Parra *et al.*, 1989, Thanomsub *et al.*, 2006). The hydrophobic chain of surfactants can contain a long chain of saturated or unsaturated fatty acids, while the hydrophilic chain can contain peptides, mono-, oligo- or polysaccharides or acids (Desai and Desai, 1993, Kosaric, 2008, Lang, 2002, reviewed by Lu *et al.*, 2007, Nagórska *et al.*, 2007, Rosen and Kunjappu, 2012, Sharma, 2016). Moreover, surfactants are mostly neutral or anionic compounds that can also be grouped based on their molecular weight and mode of action. In such a situation, the low molecular weight surfactants reduce the surface tension of water/air interfaces and the interfacial tension of oil/water interfaces, while the high molecular weight surface active compounds (SACs) are mostly bioemulsifiers and are effective in stabilizing oil/water emulsions (reviewed by Banat *et al.*, 2010, Karanth *et al.*, 1999, Muthusamy *et al.*, 2008, Neu, 1996, Rosenberg and Ron, 1999). Among the classes of surfactants, cyclic lipopeptides (CLP) are the most widely studied and are mostly expressed by fluorescent pseudomonads (D'aes *et al.*, 2010, Nielsen *et al.*, 2002, Richard *et al.*, 2012, Tran *et al.*, 2008).

1.4.1 Pseudomonas spp.

The genus *Pseudomonas* is one of the most diverse bacterial genera. Their population is rapidly increasing every year, for instance in 2006, 102 species were recognised and this number increased to 118 in 2009 and 202 in 2011 (Mulet *et al.*, 2010, Olorunleke and Höfte, 2015, Özen and Ussery, 2012). To describe the relationship between species in the genus *Pseudomonas*, different approaches are employed including the use of metabolic profiling, 16S rDNA sequencing and DNA hybridisation (Bossis *et al.*, 2000, Rossello-Mora and Amann, 2001). The 16S rDNA sequencing as a common indicator allows the designation of a strain to the genus and permits evaluation amongst very different bacteria (Santos and Ochman, 2004). However, the tenacity of 16S rRNA gene sequences at species level is low and may need to be complemented when identifying strains to species level (Anzai *et al.*, 2000, Hilario *et al.*, 2004, Moore *et al.*, 1996, Yamamoto *et al.*, 2000)**.** The metabolic activities of *Pseudomonas* spp. differ, but are generally Gram-negative, catalase positive bacteria that are abundant in the environment and utilise a wide range of carbon sources including hydrocarbons, proteins and fats (Cameotra and Makkar, 1998, Ron and Rosenberg, 2001, Wu *et al.*, 2011). The vast metabolic pathways of pseudomonads allow production of a number of active compounds, including surfactants that can facilitate a number of different biological roles (Bogino *et al.*, 2013, reviewed by Bouchez Naïtali *et al.*, 1999, Georgiou *et al.*, 1992, Soberón-Chávez *et al.*, 2005). Other bacteria groups known to express surfactants include the *Bacillus, Burkholderia* and *Aeromonas* spp. (Bicca *et al.*, 1999, Deshpande and

Daniels, 1995, Ilori *et al.*, 2005, Kim *et al.*, 2004, Sheppard and Mulligan, 1987) - see Table **1.1** for a list of some pseudomonads-related surfactant lipopeptides.

To date, the most widely studied surfactants are the cyclic lipopeptides expressed mostly by the *Pseudomonas* spp., because of their diversity and potentiality within different fields of biotechnology (Abalos *et al.*, 2001, Deziel *et al.*, 1996, Gautam and Tyagi, 2006, Itoh *et al.*, 1971, Mavrodi *et al.*, 2006, Nielsen *et al.*, 2002, Nielsen and Sorensen, 2003, Nybroe and Sørensen, 2004, Raaijmakers *et al.*, 2006, Rahman and Gakpe, 2008, Tran *et al.*, 2007).

Table 1.1 Pseudomonas related lipopeptides

Organisms	Surfactants	Reference
P. corrugate NCPPB 2445	Corpeptin	(Emanuele *et al.*, 1998)
P. corrugate IPVCT 10.3	Cormycin	(Scaloni *et al.*, 2004)
P. entomophila L48	Entolysin	(Vallet-Gely *et al.*, 2010)
P. fiuorescens DR54	Viscosinamid	(Nielsen *et al.*, 1999)
P. fluorescens SS101	Massetolid	(Tran *et al.*, 2007)
P. fluorescens BD5	Pseudofactin	(Janek *et al.*, 2010)
P. fluorescens 96.578	Tensin	(Nielsen *et al.*, 2000)
*P. fluorescens Pf*A7B	Viscosin	(Braun *et al.*, 2001)
P. fluorescens BRG100	Pseudophomins	(Quail *et al.*, 2002)
P. libanensis M9-3	Viscosin	(Saini *et al.*, 2008)
P. nitroreducens TSB.MJ10	Lipopeptide	(de Sousa and Bhosle, 2012)
P. putida BW11M1	Xantholysin	(Li *et al.*, 2013)
P. putida PCL 1445	Putisolvin	(Kuiper *et al.*, 2004)
P. syringae pv. tomato DC3000	Syringafactin	(Berti *et al.*, 2007)
P. syringae pv. Syringae	Syringomycin	(Anselmi *et al.*, 2011)
P. tolaassii	Pseudodesmin	(Sinnaeve and Michaux, 2009)
Pseudomonas spp. DF41	Sclerosin	(Berry *et al.*, 2012)
Pseudomonas spp. WJ6	Surfactin, iturin and fengycin	(Xia *et al.*, 2014)

(Source: Mnif and Ghribi, 2015)

1.4.2 Cyclic lipopeptide (CLP)

The cyclic lipopeptides (CLPs) class is amongst the most widely studied surfactants. They consist of a lipid attached to a polypeptide chain and are expressed by many members of *Bacillus* and fluorescent pseudomonads (D'aes *et al.*, 2014, Dexter and Middelberg, 2008, reviewed by Raaijmakers *et al.*, 2006). Surfactants belonging to this class include viscosin, syringomycin, amphisin and tolaasin (Figure **1.1**), which are important in defining antagonistic relations between surfactant expressing strains and other organisms, including bacteria, fungi, and viruses (Olorunleke and Höfte, 2015, Raaijmakers *et al.*, 2006). For example, some reports suggest a practical role for CLPs in providing biological control of plant root pathogenic fungi and oomycetes (De Souza *et al.*, 2003, Nielsen *et al.*, 2002).

The synthesis of CLPs in *Pseudomonas* spp. is governed by a large multimodular nonribosomal peptide synthetase (Doekel and Marahiel, 2001, Marahiel *et al.*, 1997, Mootz *et al.*, 2002) that is influenced by a number of environmental factors, including carbon sources and iron limitations (Gross, 1985, Nielsen *et al.*, 1999). The surfactant biosynthetic genes of *Pseudomonas* spp. including genes for viscosin, syringomycin, arthrofactins, orfamide and massetolides, have been fully sequenced (Berti *et al.*, 2007, de Bruijn *et al.*, 2007, Dubern *et al.*, 2008, Feil *et al.*, 2005, Gross and Loper, 2009, Paulsen *et al.*, 2005, reviewed by Raaijmakers *et al.*, 2010, Vallet-Gely *et al.*, 2010). The synthesis of surfactants in *Pseudomonas* spp. is regulated by

master switch GacA/GacS regulatory components, with mutation in any of the systems possibly resulting in loss of surfactant production (de Bruijn *et al.*, 2007). However, little information is available about the regulatory network that triggers the surfactant biosynthesis (De Souza *et al.*, 2003, Heeb and Haas, 2001, reviewed by Raaijmakers *et al.*, 2010). The second regulatory pathway known as quorum sensing has identified three regulatory genes for lipopeptide biosynthesis in *P. fluorescens* SS101 via LuxR-type transcriptional regulation moving around the *massA*, *massB* and *massC* biosynthetic genes (Song *et al.*, 2014). Studies show that there may be involvement of N-acyl homoserine lactones (N-AHLs) in the regulation of viscosin and putisolvin biosynthesis in some *Pseudomonas* spp.; however, not all *Pseudomonas* spp. require N-AHLs in surfactant production (Song *et al.*, 2014).

Investigations of metabolic profiles of *Pseudomonas* spp. indicate that a single strain can simultaneously express different CLPs with several structural similarities of one particular LP. For example, *P. fluorescens* strain SS101 produces at least eight structural analogs of massetolide A (de Bruijn *et al.*, 2008). CLPs are diverse in chemical structures and this may suggest different or perhaps multiple applications (Moffitt and Neilan, 2000, Ron and Rosenberg, 2001).

Similarly, a discussion by Rosenberg (1986) on adhesion of surfactants to hydrocarbon using emulsification of water-insoluble compounds as substrate, suggests differences in structural composition of surfactants. It clearly states that, “it

is unlikely that they all serve the same function" (Rosenberg and Kjelleberg, 1986). Haferburgh *et al.* (1986) also studied the hydrocarbon assimilation and biocide activity of surfactants and suggests a possible role in gliding and wetting of interfaces. Their study further argues that the exact function of surfactants remains unclear and that differences in producing organisms, ecosystem and the nature of substrates may influence the nature and behaviour of surfactants (Head *et al.*, 2006, Jaeger *et al.*, 1994), and that this is of great importance in identifying novel surfactants for biotechnology.

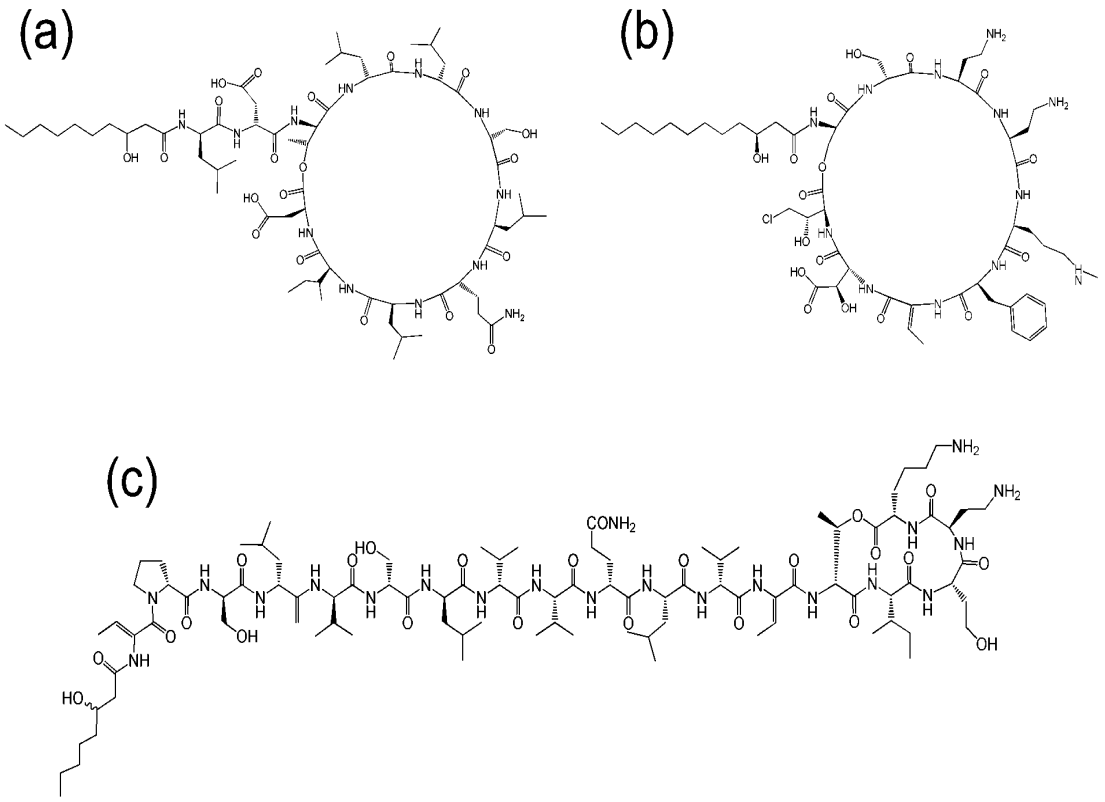

Figure 1.1: Chemical structure of lipopeptides expressed by pseudomonads (a) Amphisin (b) syringomycin (c) tolaasin I. (source: Hamley, 2015)

1.6 Thesis aims and objectives

1.6.1 Study rationale

A number of studies have focused on the isolation and characterisation of single or multiple surfactant-expressing strains for particular applications (Elazzazy *et al.*, 2015, Gudina *et al.*, 2010, Ochsner *et al.*, 1995, Pereira *et al.*, 2013, Singh and Tiwary, 2016, Velraeds *et al.*, 1996, Yadav *et al.*, 2016). The process of identifying surfactants for biotechnology starts with a survey of a large collection of bacterial isolates that are screened using high-throughput techniques such as drop-collapse assays, blood haemolysis or oil sprays to identify surfactant expression, although these techniques can give false positive results (Płaza *et al.*, 2006, Rasooli *et al.*, 2016, Youssef *et al.*, 2004). Moreover, these techniques can only classify isolates as surfactants, with no information about the producing strains. As a result, a number of different biochemical or metabolic growth-based assays and molecular-based techniques are needed to identify and characterise surfactants expressing bacteria, and this forms the basis of the research reported in Chapter 3 (Isolation and identification of surfactants expressing *Pseudomonas* spp. from soil).

In addition to high-throughput techniques that screen isolates based on qualitative assessment, a quantitative tensiometry assay is carried out to confirm and reveal surfactant strength. In this respect, several independent and bacterial surveys of surfactants have reported a limit to bacterial surfactants strength to be 24 mN/m

(Benincasa *et al.*, 2004, Fechtner *et al.*, 2011, Meylheuc *et al.*, 2001, Mohammed *et al.*, 2015, Nitschke and Costa, 2007). It is noted that recent articles have not reported any stronger surfactants (Elshikh *et al.*, 2017, Lotfabad *et al.*, 2017, Radzuan *et al.*, 2017, Rodríguez-López *et al.*, 2017). The mechanism preventing the expression of stronger surfactants currently lacks detailed explanation, even though Mohammed *et al.* (2015) have suggested the limit may have a biological or biochemical explanation.

Moreover, in any bioprospecting survey, surfactants with high activity are generally selected for testing in biotechnology and as a result, strains found to be positive for surfactant expression are investigated using quantitative surface activity measurement to find the best strains for future testing and to investigate their limit. This forms the basis of the research reported in Chapter 4 (Pseudomonads surface tension and the prediction of minimum limit).

Similarly, surfactants with high surface activity are normally characterised using different techniques such as HPLC, thin layer chromatography and NMR, MS-MS to establish their chemical and structural composition (Balan *et al.*, 2017, reviewed by Biniarz *et al.*, 2016, Varjani and Upasani, 2016). Although these techniques have high precision power, they are often expensive and thus impractical for bioprospecting studies (reviewed by Biniarz *et al.*, 2016, Kumar *et al.*, 2016). In this thesis, surfactant chemical structural diversity is studied using different oil-water behaviours including

emulsion indices, foam stabilisation and oil displacement across a number of pH and salt concentrations. This is to establish behavioural diversity among strains and assess their potentiality in different applications of biotechnology using vegetable, diesel, mineral and used lubricating oils (ULO). This forms the basis of the research reported in Chapter 5 (Diversity in surfactant behaviour).

Thus, the main aim of this research is to investigate the range of behaviours of surfactants produced by *Pseudomonas* spp., with the long-term goal of identifying novel surfactants with biotechnological applications in bioremediation, food and medical biotechnology (e.g. identification of surfactant with use in single or multiple applications).

1.7 Clarification of Aims and Objectives

Chapter 3: Isolation and identification of surfactant-producing Pseudomonas spp. from soil

I. To isolate *Pseudomonas* spp. from Dundee Botanic garden soil and to screen strains for surfactant production using the drop-collapse assay;

II. To investigate the diversity of key strains using phenotypic and growth-based assays;

III. To characterise selected strains at species level using metabolic profiles (API 20e) and 16S rDNA sequencing.

Chapter 4: Pseudomonads surface tension and the prediction of minimum limit

I. To determine surface tension of cell-free King's B (KB) medium cultures of pseudomonads that were found to be positive for the drop-collapse assay using quantitative tensiometry;

II. To identify pseudomonads that significantly reduce surface tension of liquid media and to choose key strains for further analysis;

III. To predict minimum liquid surface tension-reducing ability (LSTRA) of strains using Individual Distribution Identification (IDI) analysis;

IV. To determine effects of growth media on surfactant activity and minimum limit.

Chapter 5: Diversity in surfactants behaviour

I. To determine the behaviour of surfactants produced by key strains using emulsion indices, foam stability and oil-displacement assays;

II. To investigate the effect of buffer chemistry (pH and salt) on surfactant behaviour;

III. To investigate surfactant chemical and structural diversity from simple behaviour assays data (i and ii above) using HCA (Hierarchical Cluster Analyses) and the General linear model (GLM);

IV. To further examine the effect of media and other growth components on surfactant behaviour using quantitative tensiometry of semi-purified surfactants within a range of pH and salt concentrations.

Chapter 2

Materials and methods

2.1 General microbiology

The compositions of the media used in this research are listed in Table **2.1.** Petri dish plates were prepared using 1.5% (w/v) technical agar (Oxoid, UK), and where supplements such as antibiotics were used, their type and concentration are clearly described. All prepared media were sterilised by autoclaving at 121^{o}C for 15 min.

Bacterial liquid cultures were consistently grown in 30 ml glass universal vials (Fisher Scientific, UK) containing 6 ml of broth media. Bacterial strains were routinely grown at 20 ± 2^{o}C in a Stuart Orbital Incubator S150 bench-top shaker operating at 160 - 200 rpm. Bacterial stocks were frozen at -80^{o}C in 15 % (v/v) glycerol and were recovered by re-streaking unto plates, grown overnight/24 h at 20 ± 2^{o}C, before use. Unless otherwise stated, all experiments were conducted in four replicate (n = 4). All chemicals and media used were obtained from Fisher Scientific, UK and Oxoid, UK if not stated otherwise.

Table 2.1: The composition of media used for culturing bacteria

Media	Composition (per litre), Reference / Source
Modified King's B (KB*)	10 g glycerol, 1.5 g K_2HP0_4, 1.5 g $MgSO_4.7H_20$ and 20 g Protease peptone (Oxoid, UK) (King *et al.*, 1954). The media was modified by replacing Protease Peptone No. 3 with Protease Peptone.
Luria-Bertani (LB) medium	10 g Tryptone, 10 g yeast extract (Merck, Germany) and 10 g NaCl (Sambrook *et al.*, 1989).
Minimal M9 Medium	33.9 g $Na_2HPO_4.7H_2O$, 15 g KH_2PO_4, 2 mM $MgSO_4$, 2.5 g NaCl, 5 g NH_4Cl, 0.1 mM $CaCl_2$ and 20 mM glucose or sucrose (Sambrook *et al.*, 1989).
Nutrient media	Supplied by Oxoid, UK.
Mueller-Hinton Agar	Supplied by Oxoid, UK.
Pseudomonas Selective Agar (PSA)	*Pseudomonas* selective agar plate with CFC (cetrimide, fusidin, and cephaloridine) supplements (Oxoid, UK).
Milk plates	20 g milk powder (Tesco, UK), 1.5 g yeast extract (Merck, Germany) and 15 g technical agar (Oxoid, UK).
Tributyrin plates	20 g Tributyrin agar (Sigma-Aldrich, UK), 10 g 1, 2, 3 – Tributyrylglycerol (Sigma-Aldrich, UK).

Table 2.2: List of strains used in this research

Strains	Description	Source
Soil isolates (Strains 1-58, and Controls 1-20)	Strains isolated from Botanic Garden soil	this work

Strain 1-58 are surfactant-expressing strains

Controls are the non-surfactant-expressing strains

Table 2.3: Primers used in this research to amplify and isolate 16s rDNA

Name	Sequence (5′-3′)	Target	Source/Reference
Forward	GGTCTGAGAGGATGATCAGT	16S rDNA	(Widmer *et al.*, 1998)
Reverse	TTAGCTCCACCTCGCGGC	16S rDNA	(Widmer *et al.*, 1998)

Table 2.4: Genebank accession numbers of 16S rDNA sequence used in this research

Strains	Genome accession number
P. aeruginosa PA1	CP004054.2
P. aeruginosa PAO1	AE004091.2
P. fluorescens Pf0-1	CP000094.2
P. fluorescens NBRC 14160	NR_113647.1
P. fluorescens ATCC 13525	NR_114476.1
P. fluorescens SBW25	AJ310393.1
P. putida KT2440	AE015451.2
P. syringae ICMP 3023	NR_117820.1
P. syringae pv. tomato str. DC3000	NR_074597.1

2.2 Sample collection

In order to isolate rhizosphere associated pseudomonads, soil samples were collected from Dundee Botanic Garden located at geographical coordinates 56°27'21.7" N and 3°01'09.0" W. The garden is the property of the University of Dundee, Scotland, UK. Soil samples were obtained during five visits to the garden and collected from adjacent to the following trees: Cornelian Cherry (*Cornus Mas*), Hance (*Hemiptelea Davidii*), Moroccan Cypress (*Cupressus Atlantica*), Scots Pine (*Pinus Sylvestris*) and White Willow (*Salix Alba*).

The rhizosphere zone of the soil was collected using grid sampling technique. The soil was excavated at least 15 cm deep using a hand trowel before taking a sample in a 50ml falcon tube (Fisher Scientific, UK). Soil samples were taken to the laboratory for analysis.

2.3 Isolation of *Pseudomonas* spp. from soil

Approximately 1 g of soil sample was introduced to 20 mL of sterile deionised water. Samples were allowed to mix on an orbital shaker (Stuart rotator, SB3) at 20 rpm at an angle of 40^{o} for 18 h. Serial dilutions of the soil suspension were carried out before spreading an aliquot amount on *Pseudomonas* selection agar supplemented with cetrimide, fusidin, and cephaloridine (PSA+CFC). Plates were incubated at 20°C

for 72 h to allow colonies to grow. No more than seven colonies were selected per soil sample, and these were re-streaked on PSA+CFC agar plates to establish pure cultures before assessing isolates for surfactant expression using the drop-collapse assay.

2.4. Drop-collapse assay

In order to screen strains for surfactant expression, pure isolates recovered from Dundee Botanic Garden soil (section 2.3) were subjected to the drop-collapse assay using a modified protocol as described by Bodour and Miller-Mair (1998). 10 µL of bacterial culture were gently dropped into a sterile empty 90 mm Petri dish plate and observed after 30 s. A positive result was recorded, if the drop lost its beaded shape and spread or collapsed, and a negative result if the drop remained intact.

2.5 Phenotypic characterisation

The pure isolated strains (section 3.1) were characterised using biochemical and metabolic profiles. Some selected strains were further identified to genera using an Analytical Profiling Index (bioMerieux, UK) and 16S ribosomal DNA sequencing (16S rDNA), as described in the later following sections.

2.5.1 Appearance (fluorescence)

The key strains (section 3.1) were assessed based on their physical appearance on KB* plates and Mueller-Hinton agar. KB* and Mueller-Hinton agar plates were each drop-inoculated using 10 µL of overnight KB* broth cultures. The inoculted strains were assessed for fluorescence after 48 h incubation. A positive result was recorded if strains produced fluorescence and a negative if they did not.

2.5.2 Antibiotic susceptibility

The key strains (section 3.1) were tested for susceptibility to antibiotics using M13, M14, and M51 (MASTERING-S^{TM}) antibiotic discs. (MASTERING-S is an antibiotic ring device used to measure the sensitivity of more than six antibiotics simultaneously). Overnight KB* broth cultures were used to inoculate KB* agar plates using the spread plate technique before placing an antibiotic disc on the surface of the agar and incubating it at 20°C for 48 h. Antibiotic susceptibility was recorded based on the diameter of the zone of inhibition. A diameter of 6 mm and above was recorded as positive (and susceptible) and a value of less than 6 mm as negative (not susceptible).

2.5.3 Catalase test

The presence or absence of catalase enzyme in the key strains (section 3.1) was assessed using the method reported by Robertson *et al.* (2013). A wire loop was used

to gently mix a bacterial colony with 10 µL of hydrogen peroxide (H_2O_2). The mixture was observed after 10 s for the presence of bubbles. The presence of bubbles was recorded as a positive result and their absence as negative.

2.5.4 Gelatinase activity

Specialised gelatine plates were prepared using nutrient broth medium with 120 g/L gelatine (Dr. Oetker, UK) as described in Robertson *et al.* (2013), but with some modification. Gelatinase activity in the key strains (section 3.1) was assessed by drop inoculation of gelatine plates with 10 µL of overnight KB* broth culture. A positive result was recorded if the inoculation site turned to liquid after 48 h and negative if it remained solid.

2.5.5 Kanamycin resistance

To further confirm and study the susceptibility of strains to antibiotics, strains were re-assessed for resistance to kanamycin using a modified version of the protocol reported by Kelch and Lee (1978). Kanamycin agar plates were initially prepared using LB media supplemented with 50 µg/mL of kanamycin. Then 10 µL of overnight KB* broth culture was drop-inoculated onto each plate before incubation at 20^{o}C for 48 h. A positive result was recorded if bacterial growth was observed and plates without growth were recorded as negative.

2.5.6 KB culture acidity*

KB* broth culture acidity was assessed using the protocol described by Robertson *et al.* (2013). About 5 µL of 0.1% (w/v) bromocresol green was mixed with 50 µL of overnight KB* broth culture and observed immediately. The presence of dark colouration was recorded as a positive result and colourless or yellow colouration as negative.

2.5.7 Lipase secretion

The key strains strains (section 3.1) were assessed for the expression of lipase (an enzyme) using tributyrin plates (Sigma-Aldrich, UK). KB agar plates were drop-inoculated with 10 µL of overnight KB* broth culture and incubated at 20^{o}C for 48 h. A positive result was recorded if a clear halo zone around the colony was observed and a negative if no halo zone around the growth area was seen.

2.5.8 Mercury resistance

In order to assess the key strains (section 3.1) for mercury resistance, a modified version of the protocol reported by Komura and Izaki (1971) was adopted. In this assay, mercury plates were prepared using LB media supplemented with mercury chloride ($HgCl_2$) to make up a final concentration of 10 µg/mL. KB agar plates were drop-inoculated with 10 µL of overnight KB* broth culture before incubation at 20°C

for 48 h. Positive results were recorded if growth was observed on plates and negative if no growth was observed.

2.5.9 Mucoid secretion

The key strains (section 3.1) were assessed for mucoid appearance on KB* agar plates. Strains were streak-inoculated on KB* agar plates and were allowed to grow at 20°C for 48 h. The strains were assessed and the presence of mucoid was recorded as a positive result and its absence was recorded as negative.

2.5.10 Oxidase test

The presence or absence of cytochrome C oxidase (enzyme) in the key strains (section 3.1) was assessed using the protocol reported by Robertson *et al.* (2013). About 10 µL of 1% TMPD (N, N, N′, N′-tetramethyl-p-phenylenediamine) solution was gently mixed with a loop of a colony on a KB* agar plate. Mixtures were observed for colour changes after 10 s, and a positive result was recorded when the mixtures changed colour to blue or purple and negative when there was no colour change.

2.5.11 Protease secretion

Protease activity of the key strains (section 3.1) was assessed using specialised milk agar plates as described in Robertson *et al.* (2013). Plates were drop-inoculated with 10 µL of overnight KB* broth culture and incubated at 20°C for 48 h. A positive result

was recorded if a clear halo zone was observed around the colonies and a negative result if no halo area was seen.

2.5.12 Salt tolerance

A salt tolerance test was conducted as per Robertson *et al.* (2013) but with modification. LB agar plates were prepared with 4 % (w/v) of sodium chloride. Plates were drop-inoculated with 10 µL of overnight KB* broth culture and incubated at 20^{o}C for 48 h. Positive results were recorded if visible growth was observed after incubation and negative if there was no growth.

2.5.13 Sugar utilisation

Strains were assessed for sugar utilisation using minimal M9 Medium supplemented with 20 mM glucose or sucrose as described by Sambrook *et al.* (1989) and Robertson *et al.* (2013), but with modification. M9 agar plates were drop-inoculated with 10 µL of overnight KB* broth culture and incubated at 20°C. A positive result was recorded if growth was observed after 48 h and a negative if there was no growth.

2.5.14 Swarming motility

A swarming test was performed using the protocol reported by Robertson *et al.* (2013). Briefly, 0.5 % (w/v) agar plates were prepared using KB* nutrients. The plates were drop- inoculated with 10 µL of overnight KB* broth culture and incubated at

20°C for 48 h. Positive results were recorded if an irregular movement of colonies was observed at the tip end of the growth area, and a negative result if there was no irregular movement.

2.5.15 Swimming motility

The swimming motility characteristic of the key strains (section 3.1) was assessed using the protocol reported by Robertson *et al.* (2013). Swimming plates were prepared using 0.1x standard KB* nutrients but with 0.3 % (w/v) technical agar. The plates were stab-inoculated with 10 µL of overnight KB* broth culture, and incubated at 20°C for 48 h. A positive result was recorded if an expanded ring growth around the stab area was seen, and a negative if the growth was limited to the stab area.

2.5.16 Twitching motility

Twitching motility of the key strains (section 3.1) was evaluated using the protocol reported by Robertson *et al.* (2013). KB* agar plates were prepared but with 1.0 % (w/v) technical agar. The plates were stab-inoculated with 5 µL overnight KB* broth culture and incubated at 20°C for 48 h. A positive result was recorded if there was growth expansion between the bottom of the Petri dish and agar interface and negative if no growth was observed.

2.5.17 Temperature tolerance

Temperature sensitivity of the key strains (section 3.1) was assessed using the procedure described by Robertson *et al.* (2013). Strains were cultured in universal vials containing 6 mL of KB* broth at a temperature of 42°C for 24 h, and the strains were allowed to recover at 28°C for 24 h. The growth was assessed by optical density (OD_{600}) using a using Spectronic Helios Epsilon spectrophotometer (Thermo Fisher Scientific, UK) with 10 mm optical-path cuvettes.

2.6. Analytical Profiling Index (API 20e)

Selected strains were further characterised using the Analytical Profiling Index (bioMerieux, UK). Manufacturer's protocol was adopted when using the API kit. Similarly, the profile obtained were checked on the manufacturer's database to identify isolates.

2.7. DNA extraction

Genomic DNA was extracted from a 5 mL overnight culture of each of the key strains, using a genomic extraction kit (Bioline, UK) according to manufacturer's protocol, and was stored at -20°C for further analysis.

Similarly, plasmid DNA was extracted from a 5 mL overnight culture of each of the transformed *E. coli* strains, using a plasmid extraction kit (Bioline, UK) according to manufacturer's protocol. The plasmid DNA was stored at -20°C for further use.

2.8 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was used for cloning and analysis. The forward and reverse primers known for 16S rDNA gene (Table **2.3**) were used to amplify the target gene. A typical PCR experiment was performed in a volume of 20 µL in a 200 µL PCR tube, with each reaction containing 1µL of (10pmol) forward and reverse primer each of 10µL of 2x master mixed (Bioline, UK), 1µL of template DNA and 7µL of nuclease-free water.

PCR reaction was carried out using S1000^{TM} thermal cycler (Bio-Rad, USA). Depending on the protocol and the nature of the PCR required, the PCR program started with a denaturation step at 95°C. Subsequently, it was followed by 30 cycles consisting denaturation at 94°C, annealing step at 65°C and extension at 72°C. The PCR reaction product was analysed by gel electrophoresis.

2.9 Agarose gel electrophoresis

Agarose powder (Bioline, UK) was dissolved in 1.0x TBE buffer (Gibco, UK) to a final concentration of 0.8 % (w/v). The mixture was fully dissolved using a microwave (Daewoo, UK). About 5-10 µL of GelRed (Biotium, USA) was added before casting. The DNA sample was mixed with 6X loading buffers (Fermentas, UK) before loading.

The gels were run at 120 V for 45 – 60 minutes before visualising using a UV-light transilluminator (Bio-Rad, USA). A 1 kb DNA ladder (Fermentas, UK) was used as standard and in assessing the nature and size of the PCR product obtained.

2.10 Cloning techniques

2.10.1 Cloning

A Topo cloning kit (Invitrogen, UK) was used to clone the PCR fragment obtained for strains 1 – 5, 10, 15, 20, 25, and C1. Each of the fragments were cloned into pCR 2.1 TOPO cloning vector. The ligation reaction was used to transform competent *E. coli* DH5α. (Invitrogen, UK). The transformed *E. coli* DH5α were selected on LB plates supplemented with 100 µg/mL ampicillin plates, 20 mg/mL X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Thermo Scientific, UK) and 100 mM IPTG solution (Isopropyl β-D-1-thiogalactopyranoside) (Thermo Scientific).

The colonies developed were analysed individually by plasmid mini prep, and plasmid DNA was digested using EcoR1 enzymes (Bio-Labs, UK) and further analysed by gel electrophoresis to confirm insertion of PCR product before preparing 1:3 dilutions of the prepared plasmid and sending for sequencing.

2.10.2 Sequence analysis

DNA sequencing was conducted at DNA Sequencing and Services (MRC I PPU), College of Life Science, University of Dundee, UK using Applied Biosystems Big-Dye Ver 3.1. The sequence data obtained were first extracted and analysed using Chromas Lite 2.1 software (Informer Technologies, Inc.) before further sequence analysis with BLAST software and database tool (NCBI; National Center for Biotechnology Information, USA) to identify the potential organisms.

Moreover, multiple sequence alignment was conducted using the EMBL-EBI tools by aligning the sequence obtained from the 16S rDNA of the nine selected key strains with nine other well-studied *Pseudomonas* spp. strains (**Table 2)** and a phylogenetic tree was generated.

2.11 Foaming assays

The behaviour of surfactants in stabilising foam was examined using the protocol reported by Reiling *et al.* (1986). Usually, 18 h overnight KB broth cultures were

prepared using 6 mL microcosm and the microcosms were vortexed for 30 s to produce foam. The initial foam height (H_i) was measured immediately after vortexing. The microcosms were allowed to stand for 2 h, after which the final foam height (H_f) was measured. The reduction in foam was calculated using the formula:

$$\Delta H = \frac{(Hi - Hf)}{Hi} * 100$$

2.12 Emulsion assay

Emulsion formation was assessed using a modified version of the protocol reported by Cooper and Goldenberg (1987). About 5mL of diesel, 5mL of sterile deionised water and 5 mL of culture supernatant (n = 3) were vortexed vigorously for two minutes, and the resulting mixtures were allowed to stand for 24 h. The heights of emulsion, aqueous and oil were used to calculate corresponding percentage emulsion, aqueous and oil indices using the fomular below:

$$E24 = \frac{Height\ of\ emulsion\ (mm)}{Total\ height\ (mm)} * 100$$

2.13 Oil-displacement assays

The oil-displacement assay was performed using the procedure described by Morikawa *et al.* (1993). The assay is based on the dispersion of oil layer by surfactants. In order to assess the key strains (section 3.1), a thin oil layer was prepared by adding 10 µL of sterile used lubricating oil, diesel or mineral oil, and 100 µL of vegetable oil was put into a Petri dish containing 40 ml of deionised water. Then 10 µL of the overnight broth culture was gently dropped at the centre of the thin oil layer to form a clear zone. The diameter of the clear zone was recorded.

2.14 Partial purification of surfactants

The surfactant was semi-purified using a modified version of the protocol reported by Song *et al.* (2015). Isolates were grown in broth overnight at 28^{o}C. The cultures were then twice centrifuged at 4000 rpm for 20 min at 4°C. Culture supernatants were first filtered using 0.2m pore syringe filters (Thermo Scientific, UK) before acidifying to pH 2.0 with 9% HCl. The acidified supernatants were kept at a low temperature on ice for one hour before they were re-centrifuged at 4000 rpm for 30 min at 4°C to collect the precipitate. The precipitate was washed using acidified water of pH 2.0, and the pH was adjusted to pH 8.0 using 0.2M NaOH before re-suspending in different buffers and salt concentrations.

2.15 Surface tension measurement

The liquid surface tension was measured using the protocol described in Koza *et al.* (2009) and Fechtner *et al.* (2011). To determine the liquid surface tension, a replicate of 18 h overnight KB* broth culture (n=4) was centrifuged at 4000 rpm for 15 min to provide cell-free culture supernatant. The surface tension of the culture supernatant was measured with a Krüss K100 Mk2 Tensiometer at 20°C using the standard rod method. Using this approach, the surface tension of water was 73 ± 1 mN/m and the sterile KB* broth medium was 54 mN/m.

2.16 Prediction of minimum liquid surface tension-reducing ability (LSTRA)

In order to predict the minimum liquid surface tension-reducing ability of strains, the data generated from quantitative tensiometry of the 46 strains that significantly reduce the liquid surface tension of sterile media were examined using Individual Distribution Identification (IDI) analysis. The Anderson-Darling (AD) Goodness of Fit Test in MINITAB (MINITAB v.15, Minitab Ltd, UK) was used to identify the best theoretical probability that best fit the data and predicted minimum threshold parameters for the fitted distributions (Fechtner *et al.*, 2011, Mohammed *et al.*, 2015).

Chapter 3

Isolation and identification of surfactant-producing *Pseudomonas* spp. from soil

Preface

Bacteria, especially members of the genera *Bacillus* and pseudomonads express surface active compounds that are useful in biotechnology. Studies have shown that surfactant-expressing strains are rapidly isolated from both soil and water environments that are either contaminated or uncontaminated. In this Chapter, bacterial strains were isolated from Dundee Botanic Garden soil using media that select only pseudomonads. The isolates where screened for liquid surface tension reducing ability (LSTRA) using the drop-collapse assay before characterising the key strains using different metabolic and growth-based assays. Hierarchical cluster analysis of phenotypic characterisation data, Initial Metabolic Profiling (API 20e) and 16S rDNA sequence analyses confirmed that these strains were a diverse group of pseudomonads.

3.1 Introduction

Bacteria play a significant role in the soil ecosystem by participating in nutrient cycles and microbial interactions (Agaras *et al.*, 2015). Some species help in promoting plant growth/health, while others cause diseases (reviewed by Raaijmakers *et al.*, 2009). Amongst the beneficial bacteria, *Pseudomonas* spp. or pseudomonads are identified as plant probiotics due to their active root-colonising behaviour and production of compounds that stimulate plant growth/protection (Mercado-Blanco and Bakker, 2007). Examples of non-pathogenic species found on the ground include *P. chlororaphis*, *P. fluorescens*, *P. putida* and *P. stutzeri* (Haas and Défago, 2005) and the plant pathogens *P. cicchorii*, *P. savastanoi* and *P. syringae* (Peix *et al.*, 2009). Moreover, *Pseudomonas* spp. have been found to produce compounds that aid in phosphate solubilization, phytohormone production and induced systemic resistance and production of antibiotics (Preston, 2004, Richardson and Hadobas, 1997). This behaviour in particular makes pseudomonads attractive microorganisms for research (Walsh *et al.*, 2001).

The genus *Pseudomonas* is characterised by its intrinsic genetic and physiological diversity. They are Gram-negative, motile and oxidase-positive organisms found in the air, soil and water (Peix *et al.*, 2009). Isolation and identification of bacteria belonging to the genus *Pseudomonas* can be difficult because of their intrinsic antibiotic resistance, and therefore can require antibiotic-based selective media that will allow the selection of only *Pseudomonas* spp. (Gould *et al.*, 1985).

Pseudomonas selective agar supplemented with cetrimide, fusidin and cephaloridine is recommended for isolating *Pseudomonas* spp. from soil and other environments (Mead and Adams, 1977). This media makes it easier to select *Pseudomonas* spp. with high precision; however, grouping strains to species-level may be a challenge (Yamamoto and Harayama, 1995).

Over the years, different techniques such as growth-phenotypic assays, metabolic assays, biochemical tests or 16S rDNA sequencing have been employed in differentiating and identifying bacteria belonging to pseudomonads (Andersen *et al.*, 2000). Growth-based phenotypic assays are typically required to differentiate organisms using their nutritional requirement, for instance growth on different carbon and nitrogen sources (Sandman and Ecker, 2014). This method is cheap and does not require expensive technology (reviewed by Bochner, 2009). The biochemical test also helps reveal an organism's activities, including the expression of enzymes useful for their activities (reviewed by Bochner, 2009).

Bacillus and pseudomonads produce surface-active compounds called surfactants that help them in carbon-intake and enhanced biofilm-attachment (Fiechter, 1992, reviewed by Georgiou *et al.*, 1992, Raaijmakers *et al.*, 2010, Ron and Rosenberg, 2001). Other roles include heavy metal binding, quorum sensing and antimicrobial activity (Davey *et al.*, 2003, Hamouda *et al.*, 2001, Mulligan *et al.*, 2001). In the late 1960s, surfactants were identified as hydrocarbon-dissolving agents with the potentiality for replacing synthetic surfactants (sulfonates, carboxylates and esters),

especially within the food and pharmaceutical industries. Synthetic surfactants have a toxic effect and leave behind a high residual effect that may lead to many problems including loss in biodiversity (Sáenz-Marta *et al.*, 2015).

3.2 Chapter aim and objectives

The primary aim of the research reported in this Chapter was to isolate and identify surfactant-producing *Pseudomonas* spp. from the Dundee Botanic Garden for further studies.

The objectives of the study were:

I. To isolate *Pseudomonas* spp. from Dundee Botanic garden soil and to screen strains for surfactant production using the drop-collapse assay;

II. To investigate the diversity of key strains using phenotypic and growth-based assays;

III. To characterise selected strains to species level using metabolic profiles (API 20e) and 16S rDNA sequencing.

3.3 Sample collection

3.3.1 Site description:

The Dundee Botanic Garden is located at the geographical coordinate's 56°27'21.7" N 3°01'09.0"W. The garden belongs to the University of Dundee and was established in 1970. It has two large glass houses for tropical and warm temperate plants, and its outside areas contain an extensive collection of plants from all around the world.

3.3.2 Sampling and isolation of bacteria

Soil samples were obtained during five visits to the garden and collected close to the following trees: Cornelian Cherry (*Cornus mas*), Hance (*Hemiptelea davidii*), Moroccan Cypress (*Cupressus atlantica*), Scots Pine (*Pinus sylvestris*) and White Willow (*Salix alba*), as shown in Figure **3.1**. The soil in the rhizosphere zone was obtained using grid sampling technique (grid sampling could be useful for future work where more samples are needed for further analysis).

In order to isolate *Pseudomonas* spp. from soil samples, serial dilutions were performed and aliquots were inoculated on *Pseudomonas* selection agar (PSA+CFC) plates using the spread-plate method (Figure **3.2**). At least 7 to 8 colonies were carefully selected from each plate to minimise possible biological replicates (i.e. selecting similar strains from the same soil sample), and were re-streaked on

PSA+CFC. A total of 251 bacterial strains were isolated and screened for surfactant expression using the drop-collapse assay.

Figure 3.1 Soil sampling sites. Soil samples were collected from Dundee Botanic Garden for the isolation and identification of surfactant-producing pseudomonads. Figures **A** to **D** show the sample collection points.

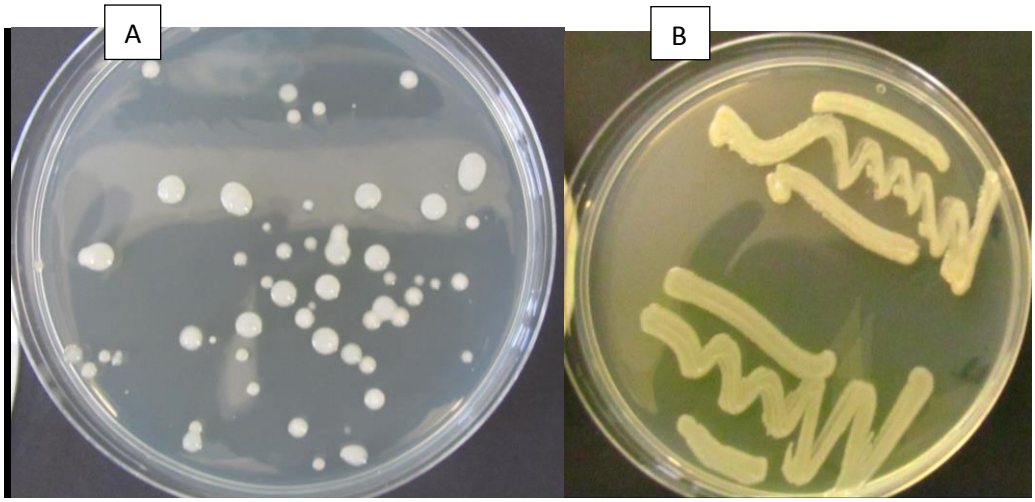

Figure 3.2 Discrete and re-streaked colonies of bacteria on PSA plates. The Dundee Botanic Garden strains were diverse in their morphology and appearance. (**A**) Discrete colonies on plates (**B**) the re-streaked plates. Petri dishes were 90 X 15 mm in diameter and height.

3.4 Screening bacterial strains for surfactant expression

Previous studies have shown the use of different techniques including haemolysis test, drop-collapse assay and oil spray in the screening and identification of surfactant-expressing bacteria (Walter *et al.*, 2010). In the course of this research, the drop-collapse assay was used to identify strains based on the expression of surfactants, before further analysis in Chapter 4.

To assay strains, aliquots of 18 overnight KB* broth cultures were placed onto the surface of a clean Petri dish. A positive result was recorded if the droplet lost its bead-like shape and spread or collapsed, while negative was registered if the drop retained its bead-like shape (see Figure **3.3**). Out of the 251 strains that were tested using this technique, 58 were found to be positive for surfactant expression (Table **3.1**). Glycerol stock of the positive strains and a set of 20 negative strains (controls) were prepared and labelled as strains 1-58 and c1-c20 before storage at -80^{o}C for further analysis.

Screening bacteria for surfactant expression using the drop-collapse assay and further characterisation using quantitative surface tension (Chapter 4) were undertaken in parallel. Data generated from the latter were used to identify 30 key strains (25 positive strains alongside of five non-surfactants expressing strains as controls) for further analysis.

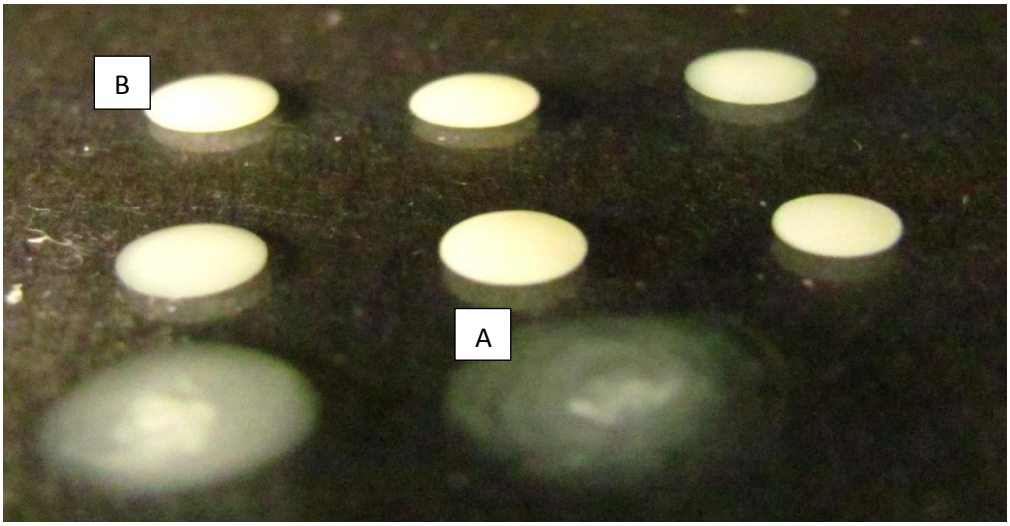

Figure 3.3 Screening strains for surfactant expression using the drop-collapse assay. Dundee Botanic Garden strains were screened using the drop-collapse assay. Aliquots of KB* broth culture were dropped onto a clean Petri dish plate and observed after 10 s. (**A**) A positive result was confirmed if the drop lost its shape and (**B**) negative if it remained static.

Table 3.1: Drop-collapse positive strains recovered from Dundee Botanic Garden soil

Visit	No. of samples collected	No. of strains isolated	No. of drop-collapse positive strains
1	8	59	15
2	8	65	15
3	8	34	8
4	8	55	5
5	8	38	15
TOTAL	**40**	**251**	**58**

3.5 Characterisation of key strains

3.5.1 Introduction

Observable properties of bacteria such as morphology, growth, appearance and biochemical characteristics are relevant parameters that can be used to differentiate between species (reviewed by Bochner, 2009). Since the publication of Bergey's manual in 1923, microbiologists have been using growth-based techniques to differentiate bacteria based upon their carbon, nitrogen, sulphur and other growth requirements (reviewed by Bochner, 2009). Other bacterial activities including enzyme secretion and antibiotic and heavy metal resistances are employed in characterising and differentiating strains.

In this research, a series of behaviour and growth-based assays used for pseudomonads after Robertson *et al.* (2013) and laboratory experience to characterise the 30 key strains were applied. Growth-based assays undertaken included swimming, swarming and twitching motilities, sugar utilisation and a siderophore test. An expression such as lipase and protease secretion, the presence of catalase, gelatinase and oxidase enzymes where also tested. Resistance to antibiotic and heavy metals was tested using an antibiotic disc, kanamycin, mercury chloride and tetracycline, while the tolerance activity was tested by KB* broth culture for acidity, salt and temperature tolerences.

3.5.2 Phenotypic characteristics of key strains

A total of 27 assays were undertaken to ascertain the phenotype of the 30 strains (see Appendix **A3.1** for strain list and phenotype). All the 30 strains (100%) tested positive for catalase and glucose utilisation, while 28 (93%) tested positive for oxidase and KB* broth culture acidity. Also 22 (73%), 26 (87%) and 18 (60%) were found to be positive for swarming, swimming and twitching motilities respectively, while 22 (73%) were positive for lipase, 26 (87%) for protease and 27 (90%) for gelatinase. Further, 12 (40%) were resistance to mercury, 2 (7%) to kanamycin and 9 (30%) to nalidixic acid. None of the strains grew on tetracycline plates.

In order to explore diversity amongst the key strains, phenotypic data were examined for similarity using Hierarchical Clustering Analysis and visualised with a constellation dendrogram, as shown in Figure **3.4**. The result indicated a significant difference between the key strains with a very low level of biological replication. It was observed that the constellation tree differentiated the key strains into two major groups, with each cluster having sub-groups. Isolates similar to each another were placed on nearby branches, thereby indicating a significant diversity amongst the key strains.

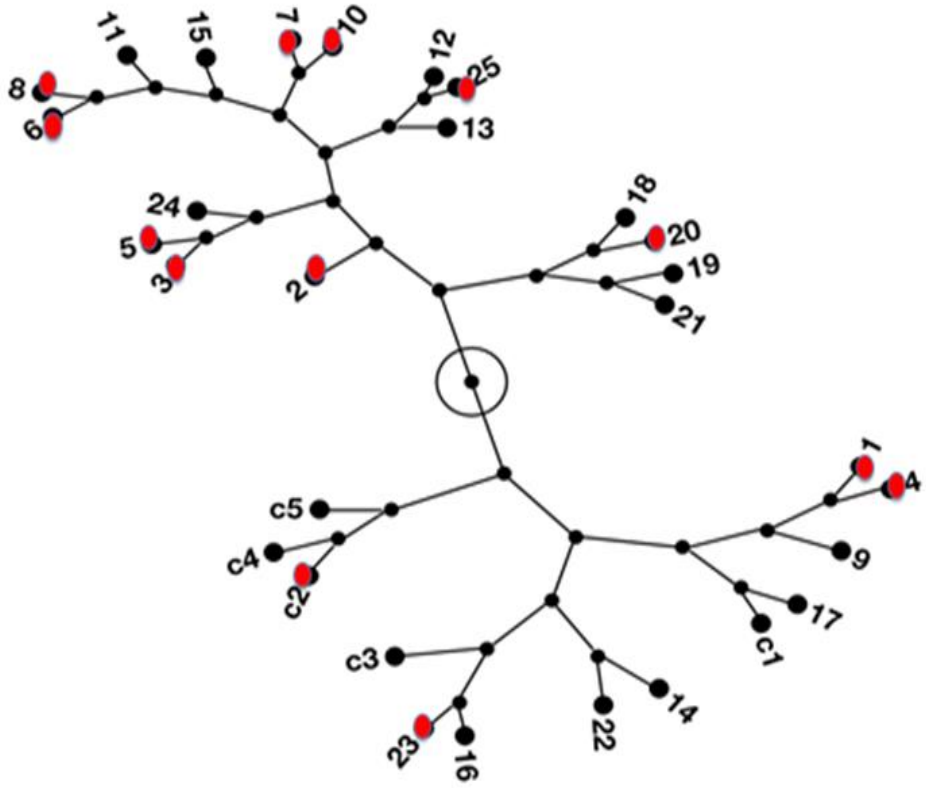

Figure 3.4 Key strains were a diverse collection of pseudomonads. The 30 key strains were assessed for similarity using Hierarchical Clustering Analysis (HCA) of the phenotypic data. Shown above is a constellation dendrogram in which the 30 key strains (1-25, c1-c5) show considerable diversity within the collection. The constellation is automatically rooted halfway along the longest branch (circled). API 20e identification of selected strains confirmed that these were pseudomonads (red circles).

3.6 Species identification

To further characterise the key strains, an Analytical Profile Index (API 20e) and 16S ribosomal DNA (rDNA) were used to identify selected strains among the 30 key strains to species level. At least 16 strains were selected from the 30 key strains and analysed using Analytical Metabolic Profiling (API 20e). Of the 16 strains tested, 12 were confirmed as pseudomonads (Table **3.2**), one as *Aeromonas salmonicida* with the API 20e database not being able to identify the remaining three.

Similarly, further characterisation of 9 strains from the 16 selected strains (i.e. strains identified using the API 20e) was achieved by isolating and cloning the 16S rDNA gene and sequencing the insert (Figure **3.5** and **3.6**). The obtained nucleotide sequences were queried on the EMBL Heidelberg database for identity and or resemblance. BLASTN confirmed the 9 strains were pseudomonads (Table **3.2**) with 99.99 % similarity (see Appendix **A3.2** for sequences).

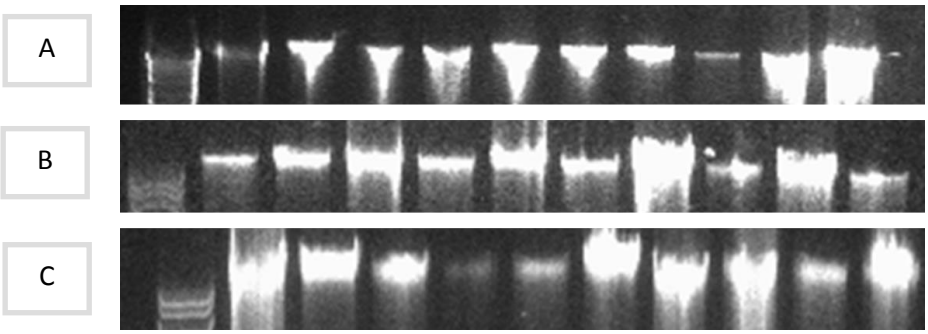

Figure 3.5 The key strains vary in genomic DNA concentration. Genomic DNA was extracted using a DNA extraction kit (Bioline, UK) for the 30 key strains (1-25, c1-c5). The image of the gel shows variation in DNA concentration. Row (A) left to right, strains 1-10; (B) left to right, strains 11-20; (C) left to right, strains 21-c5. DNA ladder is the first lane of each of the A, B and C.

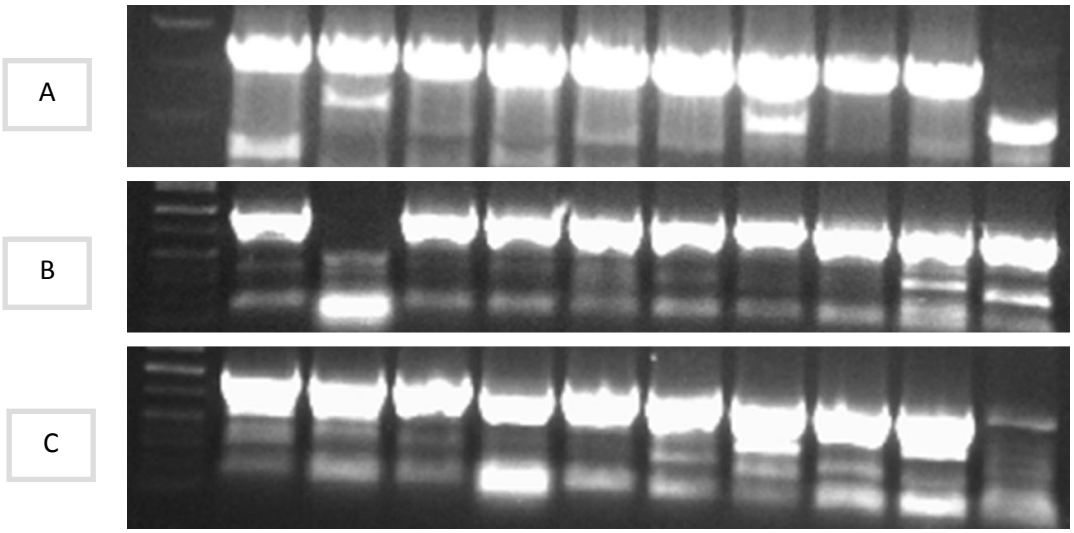

Figure 3.6 Agarose gel electrophoresis of PCR product generated by the amplification of the 16S rDNA of key strains. The genomic DNA of the 30 key strains (1-25, c1-c5) was amplified with 16S rDNA primers. Row (A) left to right, strains 1-10; Row (B) left to right, strains 11-20; Row (C) left to right, strains 21-25 and c1-c5. DNA ladder is the first lane of each of the A, B and C.

Analyses of the 16S rDNA sequences with the well-studied pseudomonads (*P. aeruginosa* PAO1, *P. aeruginosa* PA1, *P. fluorescens* NBRC 14160, *P. fluorescens* ATCC 13525, *P. fluorescens* Pf0-1, *P. fluorescens* SBW25, *P. putida* KT2440, *P. syringae* pv. tomato str. DC3000 and *P. syringae* ICMP 3023) confirmed significant similarities existed between them (Figure **3.7**), thereby indicating that the strains were members of the genus pseudomonads.

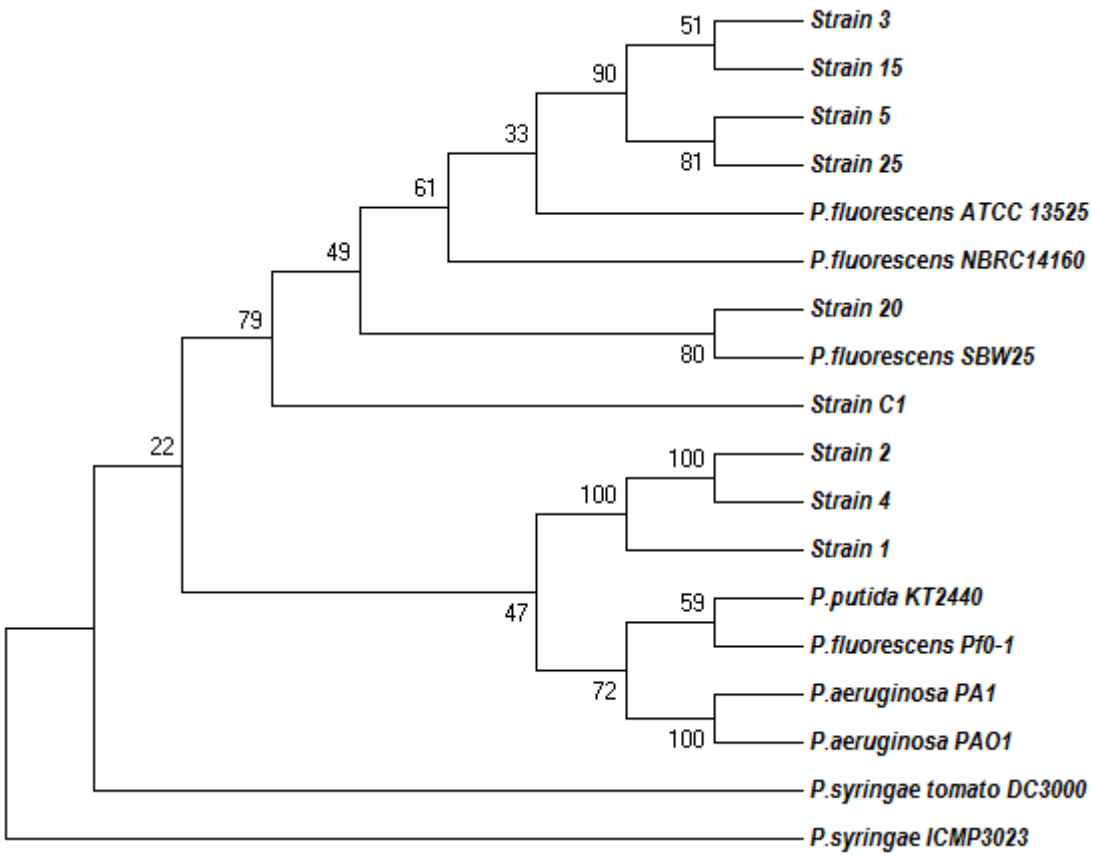

Figure 3.7: The 16S rDNA sequences of selected strains show a degree of similarity with members of the genus pseudomonads. The 16S rDNA sequence of the selected strains were subjected to multiple sequence alagnment in Mega7 software version 7.0 (MEGA, UK) with well-studied pseudomonads strains. As shown above, the sequences indicate a remarkable similarity to members of the genus pseudomonads.

Table 3.2: Strain identification using the Analytical Profile Index (API 20e) and 16S rDNA sequencing

Strain	API ID	API Sig. specie	16S rDNA Sig. specie	ID match (%)
Strain 1	2027046	*P. fluorescens/putida*	*P. helmanticensis* OHA11	99
Strain 2	2026044	*P. fluorescens/putida*	*P. trivialis* P 513/19	99
Strain 3	2136046	*P. aeruginosa*	*P. fluorescens* NBRC 14160	99
Strain 4	2226046	*P. fluorescens/putida*	*P. helmanticensis* OHA11	99
Strain 5	2326046	*P. aeruginosa*	*P. salomonii* CFBP 2022	99
Strain 6	2026006	*P. fluorescens/putida*	ND	
Strain 7	2026004	*P. fluorescens/putida*	ND	
Strain 8	2036046	*P. aeruginosa*	ND	
Strain 10	3027056	*P. luteola*	PS	
Strain 15	3127004	Unacceptable/*Bibersteinia trehalosi*	*P. fluorescens* ATCC 13525	99
Strain 20	2127006	*P. fluorescens/putida*	*P. marginalis* ICMP 3553	99
Strain 25	2126046	*P. fluorescens/putida*	*P. fluorescens* CCM 2115	99
Strain c1	4127004	Unacceptable/*Burkholderia cepacia*	*P. kilonensis* 520-20	99
Strain c2	2125006	*P. fluorescens/putida*	ND	
Strain c3	2122105	*Aeromonas salmonicida* ssp	ND	
Strain c4	7325317	unacceptable	ND	

Key:
API ID = Analytical profile Index number.
API Sig. specie = API bacterial strain as identified by the API 20e database.
16S rDNA Sig. specie = the first bacterial strain that matched the sequence-blast result.
ID match = the identity match entailed by the blast match.
ND = strain that was not identified using 16S rDNA sequencing.
PS = Poor sequence obtained.

3.7 Chapter Discussion

Soil represents a conducive environment for microbial proliferation (Kumar *et al.*, 2012). Isolation and screening of pseudomonads for surfactant expression is relatively straightforward (reviewed by Desai and Banat, 1997). However, their identification can present challenges (Yamamoto and Harayama, 1995). A total of 251 pseudomonads were isolated from Dundee Botanic Garden soil. Strains were first screened for surfactant expression to identify key strains for further investigation using different phenotypic and biochemical assays.

The results showed that the 30 key strains exhibited phenotypic characteristics similar to those of the genus pseudomonads. Moreover, further characterisation of selected strains using the API 20e kit and 16S rDNA gene confirmed strains were pseudomonads, with more than 9 species showing 99% similarity, thereby making it difficult to identify strains to species level (Bossis *et al.*, 2000, Moore *et al.*, 1996). This corresponds to a study by Yamamoto *et al.* (2000) that indicates that *Pseudomonas* spp. identification could not be resolved using 16S rDNA sequencing alone. Similarly, a review by Janda and Abbott, (2002), shows that all methods used to classify bacteria to species level have limitations because no single method can provide results that are 100% reliable. However, this research concluded that based on phenotypic characterisation and API 20e and 16S rDNA sequencing results, that the 30 key strains were members of the genus pseudomonads.

Although phenotypic characterisation was conducted to identify strains as pseudomonads, information obtained from the assays mainly enzymatic activities provided useful information in prospecting for novel applications in biotechnology. In addition, it is noteworthy that over 80% of the key strains were positive for catalase, lipase and protease secretion. These essential qualities are useful in different biotechnological industries (reviewed by Adrio and Demain, 2014); for instance, lipase secretion has been helpful in the synthesis of biopolymers and biodiesel, enantiopure pharmaceuticals, flavouring compounds and agrochemicals (reviewed by Jaeger and Eggert, 2002). Protease is useful in industries such as agrochemicals, leather and pharmaceuticals, which comprise over 60% of the global market and 25% of total worldwide enzyme sale (reviewed by Adrio and Demain, 2014). Catalase finds relevance in a range of different bioprocesses and chemical industries including personal care, pulp and paper (reviewed by Kirk *et al.*, 2002).

Hierarchical Cluster Analysis of the 30 key strains using the phenotype data indicated strains were significantly different to one another ($p < 0.001$). Moreover, a phylogenetic analysis of 16S rDNA sequences, including sequences from well-studied *Pseudomonas* spp., confirmed strains were a diverse collection of pseudomonads, and is in agreement with findings by Campbell *et al.* (1995) that showed variation amongst a group of *Pseudomonas* isolated from soil. However, although variation was observed amongst phenotypic data, a study by Smits *et al.* (2006) shows that properties may vary and expression may depend on

environmental conditions such as temperature, pH, and the nature of the substrate used.

In this Chapter, primary concerns were to recover pseudomonads from soil and to screen a diverse collection for surfactant expression using the drop-collapse assay for further analyses.

3.8 Chapter Conclusion

The work reported in this Chapter resulted in the collection of 251 strains of bacteria from the Dundee Botanic Garden, which were screened for surfactant expression using a drop-collapse assay, with 58 strains found to express surfactants. Moreover, of the 58 strains, 30 key strains (25 plus five non-surfactant expressing strains as controls) were selected and characterised phenotypically.

The phenotypic characterisation by plate-based growth assays, Initial Metabolic Profiling and 16S rDNA sequences confirmed that these strains were a diverse group of pseudomonads. The 30 key strains are further discussed in Chapters Four and Five of this thesis.

Chapter 4

Pseudomonads surface tension and the prediction of minimum limit

Preface

Surfactants are important in biotechnology. Their identification involves a survey of screened bacterial strains using a qualitative test such as the drop-collapse assay. These are later confirmed using quantitative surface activity measurement. In this Chapter, pseudomonas strains that were found to be positive for surfactant expression using the drop-collapse assay in Chapter 3 were further examined using a quantitative surface activity measurement to confirm surfactant expression. A minimum liquid surface tension-reducing ability was determined in both KB* and M9Glu cultures using an Individual Distribution Analysis, with this result being in agreement with existing literature. Finally, key strains were selected based upon their liquid surface tension-reducing ability for further study in Chapter 5.

4.1 Introduction

Growing interest in understanding surfactants' physicochemical and biological properties directly relates to their potentiality in biotechnology (reviewed by Desai and Banat, 1997, Lang, 2002, Singh and Cameotra, 2004). Surfactant expression by environmental pseudomonads has been investigated using assays that include drop-collapse, blood haemolysis, oil sprays and quantitative surface tension measurements (Bodour *et al.*, 2003, Walter *et al.*, 2010). For the latter, a strong surfactant reduces surface tension of water from 72 mN/m to a value of less than 30 mN/m (Andrade Silva *et al.*, 2014, Diniz Rufino *et al.*, 2014, El-Sheshtawy and Doheim, 2014, Liu *et al.*, 2015, Souza *et al.*, 2014).

Considerable attention is being placed on surfactant surface/interface activity. This is perhaps because most biological activity occurs at interfaces and is regulated by molecule structure and physical state at interfaces (Infante and Moses, 1994, Kaiser and Kezdy, 1984). This explains various bacterial processes including biomembrane transport, adsorption and assimilation of nutrients and toxic compounds (AlAbbas *et al.*, 2012, Liu *et al.*, 2015). Surface activity measurement has been useful in evaluating surfactant-producing strains, with the first surfactant to be characterised (surfactin - expressed by *Bacillus subtilis* S 499) for reducing surface tension of water from 72 mN/m to 27mN/m (Peypoux *et al.*, 1994). Consequently, surfactants with higher activity have not been reported, despite many surveys reporting surface activity from 22-30 mN/m (Amani *et al.*, 2013, De Lima *et al.*, 2009, Diniz Rufino *et*

al., 2014, El-Sheshtawy and Doheim, 2014, Saimmai *et al.*, 2012, Silva *et al.*, 2010, Whang *et al.*, 2008).

A recent study by Fechtner *et al.* (2011) evaluates liquid surface tension-reducing ability (LSTRA) of environmental pseudomonads using Individual Distribution Identification Analysis, and predicts a minimum threshold for bacterial surfactants to be 24 mN/m. Mohammed *et al.* (2015) confirm this prediction by screening and characterising a large collection of surfactant-expressing pseudomonads recovered from wastewater and contaminated sludge. Analysis of 59 published reports does not provide any higher activity being observed by Fechtner *et al.* (2011) and Mohammed *et al.* (2015), perhaps indicating a robust limit that covers all bacterial genera rather than being specific to pseudomonads (Mohammed *et al.*, 2015).

Limit to surface activity will be a setback for both high activity surveys and applications in biotechnology. Moreover, the lack of limit mechanism studies suggests a biological constraint in the expression of stronger compounds to avoid self-damage by the producing cells. This could also be due to growth media limitations and other physicochemical interactions generating selective pressure, thereby stopping organisms from stronger surfactant production to preventing unbearable self-damage (Fechtner *et al.*, 2011, Louvado *et al.*, 2010, Mohammed *et al.*, 2015). Further research is therefore needed to establish if similar limit can be obtained in purified surfactants, based on the assumption that the limits have biological or physicochemical explanation.

4.2 Research objectives

The aim of this Chapter is to characterise strains found to be positive for the drop-collapse assay using quantitative tensiometry, and to use this data to predict minimum liquid surface tension-reducing ability (LSTRA). This information will support the identification of key strains for further analysis.

The research objectives are to:

I. Determine surface tension of cell-free KB* broth cultures of pseudomonads that were found to be positive for the drop-collapse assay using quantitative tensiometry;
II. Identify pseudomonads that significantly reduce surface tension of liquid media and to choose key strains for further analysis;
III. Predict minimum liquid surface tension-reducing ability (LSTRA) of strains using Individual Distribution Identification (IDI) analysis;
IV. Determine the effects of growth media on surfactant activity and minimum limit.

4.3 Pseudomonads surface activity

The research reported in Chapter 3 (Section **3.4**) resulted in the identification of 58 pseudomonad strains positive for the drop-collapse assays from a collection of 251 strains.

To quantify the strength of surfactant expressed by the aforementioned strains, 18 h cell-free KB* broth cultures of the 58 strains found to be positive for the drop-collapse assay plus 20 non-surfactant expressing (controls) were further examined by quantitative tensiometry using a Krüss K100 Mk2 Tensiometer. Surface tension of water and sterile KB* medium were initially measured to ensure proper functioning of the tensiometer before the test strains (see Appendix **A4.1** for the surface tension data of the 58 expressing and 20 non-expressing strains).

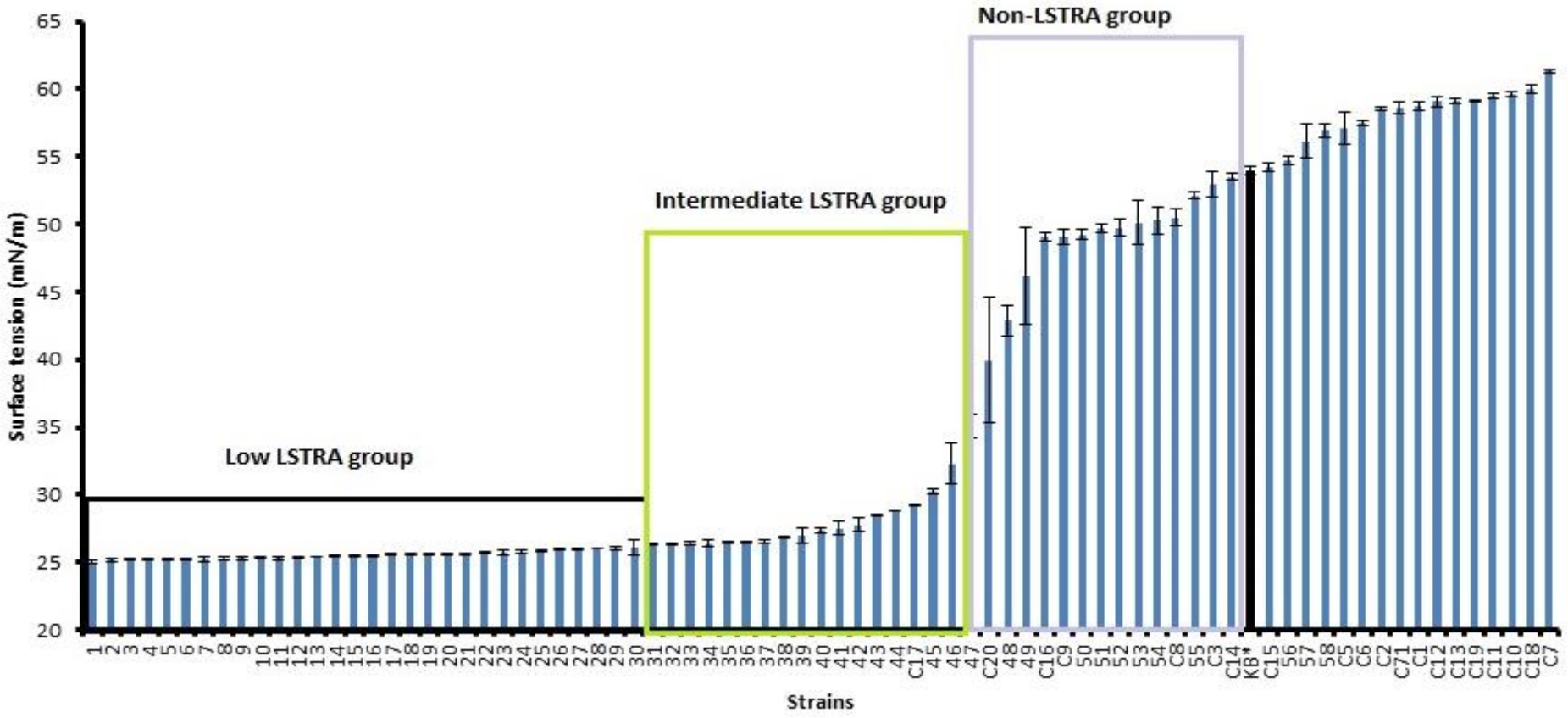
Non-LSTRA group
Intermediate LSTRA group
Low LSTRA group
Surface tension (mN/m)
65
60
55
50
45
40
35
30
25
20
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 C17 45 46 47 C20 48 49 C16 C9 50 51 52 53 54 C8 55 C3 C14 KB* C15 56 57 58 C5 C6 C2 C71 C1 C12 C13 C19 C11 C10 C18 C7
Strains

Figure 4.1: Assessing pseudomonads surface tension reducing ability. Liquid surface tension reducing ability (LSTRA) was assessed using 18 h cell-free KB* broth cultures (n = 4) by quantitative tensiometry. Shown here are the mean (± SE) surface tensions of the drop-collapse positive pseudomonad strains (strains 1 – 58), plus 20 non-surfactants expressing strains (c1 – c20) recovered from Dundee Botanic Garden. Sterile medium (KB*) is shown for reference (black bar). Dunnett's method was used to identify strains (46 of 58) that significantly reduce the surface tension (~54 mN/m to less than ~32 mN/m; $\alpha = 0.05$) and ANOVA used to examine for significant variation in LSTRA between the 58 strains (ANOVA, $p < 0.001$) The LSTRA were further differentiated into low LSTRA (strains 1-30), intermediate LSTRA (strains 31-46), and non-LSTRA (strains 46 - 58). It is important to note that the strain numbers shown were changed once the 25 key strains were identified for further analysis (see Appendix **A4.1** for detail of change).

Analysis of surface tension data for the 58 strains tested for LSTRA showed only 46 strains significantly reduced surface tension of sterile King's B (KB*) medium from ~54 mN/m to less than ~32 mN/m (Dunnett's method, $\alpha = 0.05$ using KB* as control) (Figure **4.1**). It was observed that 12 of the drop-collapse positive strains were negative for surfactant expression. This indicates that drop collapse assay is quite reliable and cannot be used as a single screening tool for surfactant expression by bacteria.

Analysis of Variance (ANOVA) of the surface tension data showed a significant difference existing between the surfactant-expressing strains ($p < 0.05$). However, *post hoc* testing of differences between the means by Tukey-Kramer HSD differentiated strains into three groups (TK - HSD, $\alpha = 0.05$) (Appendix **A4.2**). A large homogenous group composed of 30 strains having low LSTRA, 16 with intermediate LSTRA and the remaining nine made up the higher-LSTRA group with activity ranges of ~24 – 26 mN/m, 26 - 43 mN/m and 43 - 58 mN/m respectively (Figure **4.1**). This poses a weak hypothesis that the strains might be expressing more than three types of surfactants with differing surface activities.

4.3.2 Section concluding remarks

Out of the 58 strains tested for liquid surface tension-reducing ability by quantitative tensiometry, 46 strains significantly reduced the liquid surface tension of sterile KB*

medium. Strains were divided into three homologous groups, suggesting a weak hypothesis that strains might be expressing three different surfactants.

4.4 Identification of strains for further study

In bioprospecting surveys of bacteria with potential novel surfactants, those that significantly reduce surface tension of water the most are generally selected for testing for specific applications in biotechnology. In this study, *post hoc* analysis for means identified a homogeneous group of *Pseudomonas* spp. producing a limited range of very low surface activity in KB* cultures (24-26 mN/m; see Figure **4.1**). However, it is unclear how much structural and behavioural variation may exist amongst these high performing surface-active compounds.

In order to investigate behavioural diversity among the low-LSTRA group, 25 low LSTRA strains forming part of the homogeneous group (strains 1- 25 presented left to right in Figure **4.1**) were chosen alongside of 5 controls for further study in Chapter 5. The strains were re-numbered for easy conduct of the experiments, as presented in Table **4.1**. and Appendix **A4.1** and hereafter referred to as the key strains.

Table 4.1: List of key strains with their final number code and surface tension measurements in KB* and M9Glu medium

Final strain No.	Initial strain No.	ST in KB*(mN/m)	ST in M9Glu (mN/m)
1	Strain 40	25.04 ± 0.09	26.04 ± 0.13
2	Strain 34	25.20 ± 0.12	25.28 ± 0.09
3	Strain 7	25.21 ± 0.08	25.40 ± 0.19
4	Strain 42	25.23 ± 0.08	25.83 ± 0.09
5	Strain 13	25.24 ± 0.01	25.22 ± 0.05
6	Strain 15	25.26 ± 0.03	25.29 ± 0.04
7	Strain 33	25.26 ± 0.16	25.25 ± 0.10
8	Strain 36	25.26 ± 0.12	25.21 ± 0.03
9	Strain 3	25.29 ± 0.11	25.96 ± 0.01
10	Strain 31	25.30 ± 0.08	25.28 ± 0.72
11	Strain 37	25.31 ± 0.09	25.14 ± 0.03
12	Strain 14	25.32 ± 0.04	25.33 ± 0.01
13	Strain 12	25.39 ± 0.03	25.76 ± 0.08
14	Strain 23	25.43 ± 0.07	25.18 ± 0.06
15	Strain 27	25.46 ± 0.07	25.17 ± 0.06
16	Strain 20	25.51 ± 0.05	29.23 ± 0.32
17	Strain 43	25.57 ± 0.07	26.25 ± 0.06
18	Strain 30	25.57 ± 0.07	25.86 ± 0.07
19	Strain 41	25.59 ± 0.08	25.78 ± 0.04
20	Strain 52	25.60 ± 0.06	25.24 ± 0.10
21	Strain 45	25.62 ± 0.09	25.29 ± 0.10
22	Strain 29	25.71 ± 0.06	28.65 ± 0.34
23	Strain 9	25.74 ± 0.21	25.15 ± 0.06
24	Strain 32	25.77 ± 0.09	26.50 ± 0.09
25	Strain 51	25.83 ± 0.04	25.76 ± 0.07
C1	Control 1	58.77 ± 0.32	42.38 ± 3.48
C2	Control 2	58.49 ± 0.12	72.52 ± 0.35
C3	Control 3	52.95 ± 0.91	58.57 ± 1.47
C4	Control 4	58.58 ± 0.45	69.48 ± 0.05
C5	Control 5	57.13 ± 1.17	73.10 ± 0.74

ST in KB*= Surface tension of strains in modified KB (KB*) medium.
ST in M9Glu = Surface tension of strains in Minimal M9 medium supplemented with glucose.

4.5 Predicting minimum liquid surface tension-reducing ability in KB* medium

In order to predict the minimum liquid surface tension-reducing ability (LSTRA) of the strains in KB* broth cultures, surface tension data for the strains found to significantly reduce surface tension of sterile KB* medium (strains 1-46; see Figure **4.1**) were further examined to determine a minimum limit for bacterial surfactant activity in KB* cultures. This collection excluded the 12 higher-LSTRA strains because they were statistically an extension of the non-surfactant producing strains (TK-HSD, α=0.05) and the 20 non-surfactant expressing strains (controls).

Individual Distribution Identification (IDI) analysis was used to predict minimum threshold from the 16 theoretical distributions in MINITAB. The results showed that data fit 3-parameter Gamma, 3-parameter Log-logistic, 3-parameter Lognormal and Johnson transformation distributions, with the best fit provided by the 3-parameter Log-logistic distribution based on the low Anderson-Darling goodness of fit test value (AD=0.721). The minimum threshold predicted by the Log-logistic was 24.74 mN/m, which is in agreement with minimum limits predicted by the 3-parameter Gamma and 3-paramter Log-normal distribution (see Figures **4.2** and **4.3**).

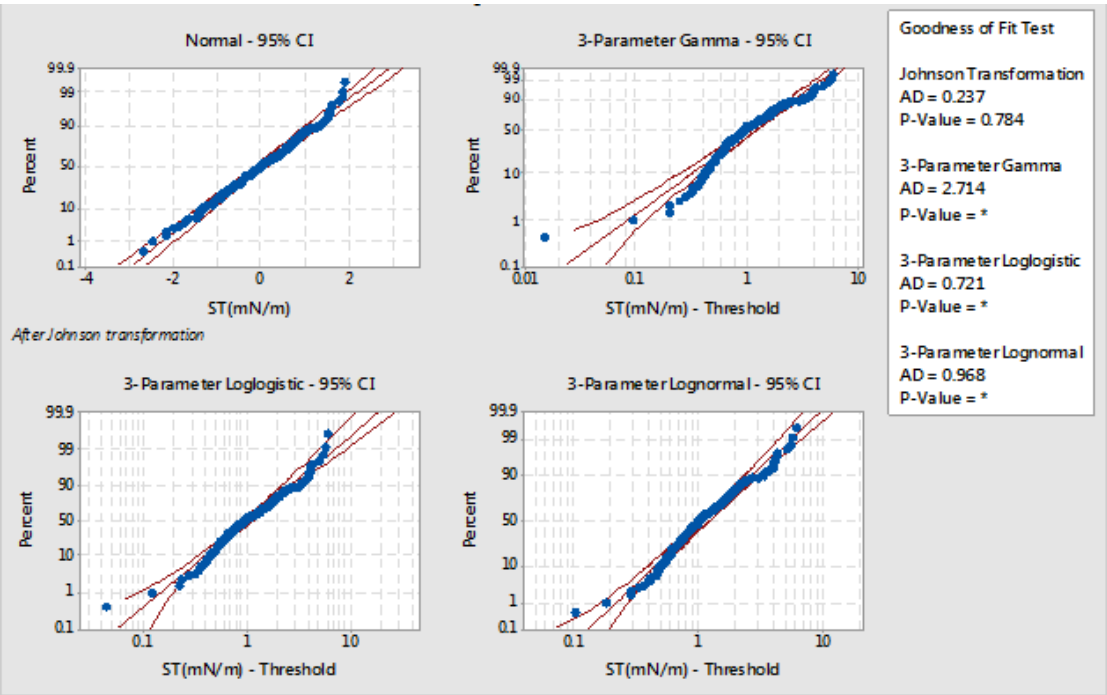

Figure 4.2: Individual distribution identification of LSTRA pseudomonad strains in KB* medium. Surface tension data for the 46 strains were subjected to 16 theoretical distributions in MINITAB. The goodness of fit graphs indicates the data fitted 3-parameter distributions (Gamma, Log-logistic, Lognormal and the Johnson transformation), with the best-fit provided by 3-parameter Log-logistic as demonstrated by the low Anderson-Darling test value (AD = 0.721). The asterisks (*) indicate p-value is impossible to calculate.

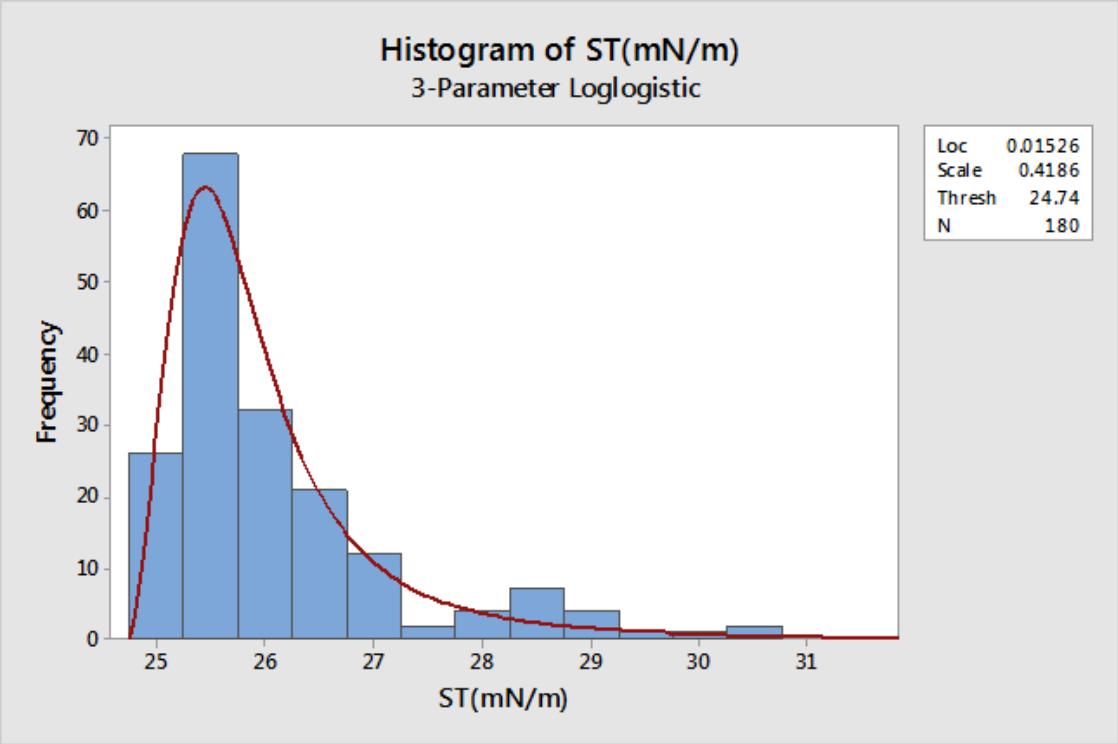

Figure 4.3: Surface tension distribution as informed by the low and intermediate LSTRA groups. Shown here is the frequency distribution of the surface tension data for the 46 strains used to predict the minimum LSTRA. The distribution was skewed to the right and the red line is the Loglogistic distribution fit to the data.

4.6 Effect of growth media on surfactant expression and minimum prediction

The key strains (30; 25 low LSTRA and 5 control strains) were investigated to assess the effect of growth media components on surfactant expression and minimum prediction. These key strains were cultured in Minimal M9Glu medium (Minimal M9Glu medium is a simple medium with few additional chemicals such as calcium and sodium salt which could interfere with surfactant activity). The cell-free M9Glu culture was analysed for LSTRA using quantitative tensiometry.

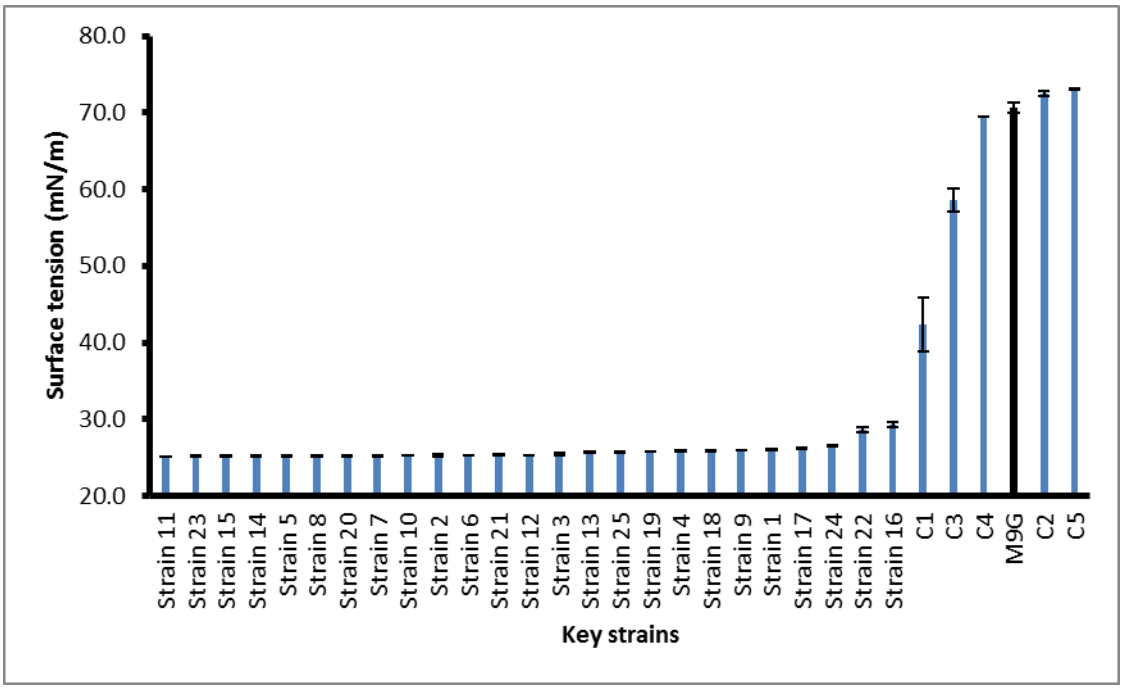

Figure 4.4: The 30 key strains show similar strength of activity in M9Glu cultures. The effect of growth media on surfactant expression was assessed using 18 hr cell-free M9Glu cultures by quantitative tensiometry. Shown above is the mean (± SE) of surface activity data of the 30 key *Pseudomonas* spp. strains (1 – 25), plus five controls that do not express surfactants (c1 – c5) (n = 5). Sterile medium (M9Glu) is shown for reference (black bar). *Post hoc* analysis classified these strains into two homologous groups (Tukey-Kramer HSD, $\alpha = 0.05$): the key and control strains.

The surface activity data of the key strains in M9Glu cultures were analysed using the approach described in the previous section (Section **4.3**). Analysis of variance between surface tension measurements showed significant difference existing between the key strains (ANOVA, $p<0.05$). Moreover, the 25 key strains significantly reduced the surface tension of sterile M9Glu medium from 70 mN/m to a value of less than 30 mN/m (Dunnett's method, α = 005 using sterile M9Glu media as control), as shown in Figure **4.4**. However, *post hoc* testing of means only identified two homogeneous groups (the key and control strains; TK-HSD, $\alpha = 0.05$).

In order to predict the minimum limit for bacterial surface activity in M9Glu cultures, the surface tension data for the 25 key strains obtained in M9Glu cultures, excluding the five controls, were analysed using Individual Distribution Identification analysis (IDI) as described in Section **4.5**). Best fitting distribution was provided by the 3-parameter Gamma distributions predicting the minimum threshold to be 24.98 mN/m (Figure **4.5**).

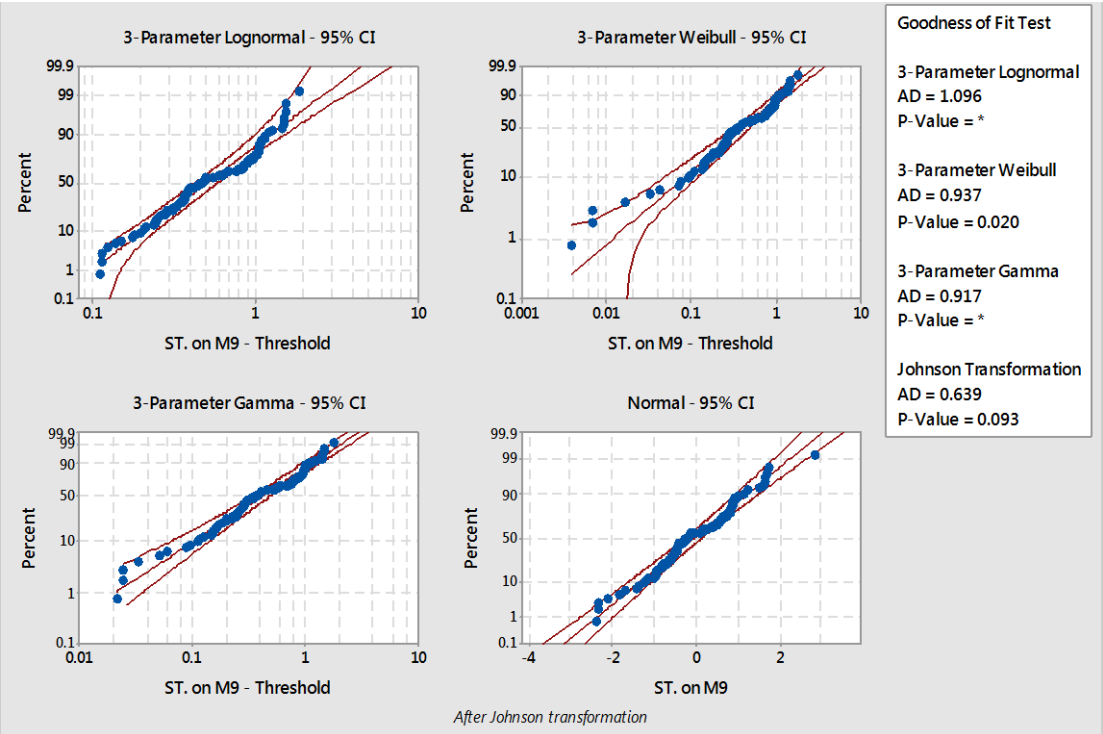

Figure 4.5: Individual distribution identification and minimum prediction in M9Glu cultures. The surface activity data for the 25 key strains in Minimal M9Glu cultures were processed using Individual Distribution Analysis in MINITAB. As shown above, the data best fit the 3-parameter Gamma based on the Anderson-Darling test value (AD = 0.917) predicting the minimum to be 24.98. The asterisks (*) indicate p-value was impossible to calculate.

4.6.1 Comment on the limits in KB and M9Glu cultures*

The effect of growth media components on surfactant expression and activity was investigated more robustly by examining surface tension measurements for the key strains in KB* and M9Glu cultures medium. The correlation analysis between the data sets showed a positive relationship (R^2 = 0.246, p = 0.014) existing between the two measurements in KB* and M9Glu media, as shown in Figure **4.6**. This thereby indicates that growth component has limited effect on surfactant expression. Although some strains showed unusual surface activity in M9Glu cultures, this did not affect surfactant expression by the strains, and could have been due to chemical interaction between surfactants and the M9Glu salts.

Investigating the limit predicted in the M9Glu cultures showed substantial agreement with the limits in KB* (this study) and existing literature (Fechtner *et al.*, 2011, Gao *et al.*, 2016, Mohammed *et al.*, 2015, Radzuan *et al.*, 2017) (Table **4.2**). Since the limit was within the reported surface activity range, it is noteworthy that theoretical limit may be due to the biology of the producing strains. These could suggest that the prediction is robust and is not affected by the chemical components present in the media.

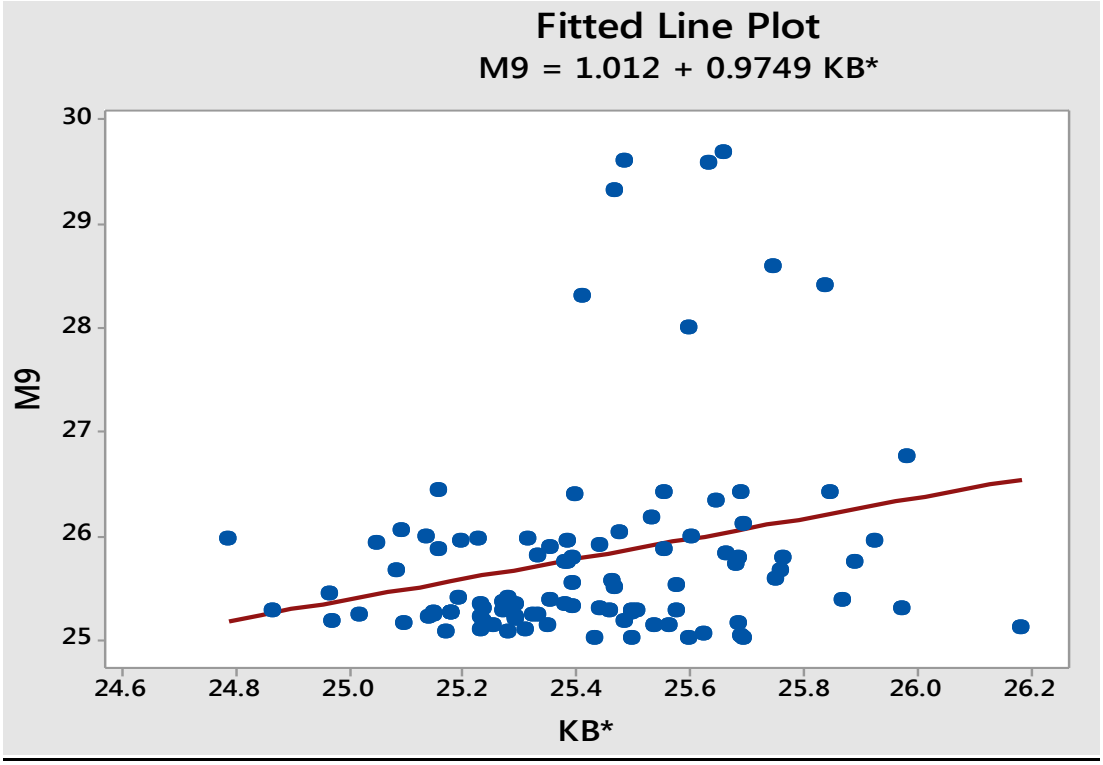

Figure 4.6: Key strains exhibit similar surface activity in KB* and M9Glu media. Surface tension measurements of the key strains obtained in KB* and M9Glu cultures were further examined by correlation analysis. As shown above, a positive correlation exists between the two measurements ($R^2 = 0.246$, $p < 0.014$). However, some strains show higher than expected activity when grown in M9Glu cultures, suggesting interference by chemical component presence in the medium.

Table 4.2: Comparison of the minimum activity limit predictions by individual distribution identification analysis (IDI)

Data set	Best-fitting 3-parameter Distribution	*N*	*P*	AD	Predicted minimum (mN/m)
Botanic Garden Pseudomonads (This study)	Log-logistic	46	*	0.721	24.74
Contaminated soil And activated sludge Pseudomonads (Mohammed *et al.,* 2015)	Log-logistic	50	0.294	0.497	24.24
Soil-isolated pseudomonads (Fechtner *et al.,* 2011)	Gamma	38	0.233	0.688	24.16

N = sample size
AD = Anderson-Darling goodness of fit test value
The asterisks (*) indicate p-value is impossible to calculate.

4.7 Chapter discussion

Surfactant expression by pseudomonads has been studied using different methods including drop-collapse assay, oil sprays, blood agar and quantitative surface tension measurements (Bodour and Miller-Maier, 1998, Peypoux *et al.*, 1994, Walter *et al.*, 2010). Previous studies on isolation and characterisation of environmental pseudomonads have reported surfactant activity from 24 - 30 mN/m (Andrade *et al.*, 2014, De Lima *et al.*, 2009, Diniz Rufino *et al.*, 2014, Liu *et al.*, 2015, Saimmai *et al.*, 2012, Souza *et al.*, 2014).

In this chapter, 58 pseudomonad strains positive for the drop-collapse assay and 20 strains negative for surfactants expression (controls) were tested for liquid surface tension-reducing ability (LSTRA). Of the 58 strains tested, 46 significantly reduced the surface tension of sterile KB* medium. 25 key strains were chosen from the low LSTRA plus five controls for further analysis.

Analysis of surface tension data for the 46 LSTRA (in KB*) using Individual Distribution Identification analysis in MINITAB to determine minimum threshold from 16 theoretical distribution was conducted. This found data best-fit Log-logistics distribution, based on the Anderson-Darling goodness of fit test (AD = 0.237) predicting the minimum to be 24.74 mN/m. This minimum threshold was in reflection of existing literature (Al-Bahry *et al.*, 2013, Dalili *et al.*, 2015, Diaz De

Rienzo *et al.*, 2016, Elazzazy *et al.*, 2015, Gao *et al.*, 2016, Joshi *et al.*, 2015, Kügler *et al.*, 2015, Liu *et al.*, 2015, Mohanram *et al.*, 2016, Mohammed *et al.*, 2015, Radzuan *et al.*, 2017).

Investigating the effect of growth media on surfactant activity by analysing the surface tension data of the 30 key strains grown in M9Glu media has shown that the 25 key strains significantly reduced the surface tension of sterile minimal M9Glu media. This findings is in conformity with other findings that studied the effect of salt on surfactant activity (Abouseoud *et al.*, 2008, Abu‐Ruwaida *et al.*, 1991, Deziel *et al.*, 1996, Lotfabad *et al.*, 2009). Although, few strains showed a slight decrease in surface activity when grown in M9Glu cultures, it suggests possible complex formation between the M9Glu salts and surfactants and conforms with the existing findings (Elshafie *et al.*, 2015, Makkar and Cameotra, 1997)**.** However, data analysis of the measurements obtained in KB* and M9Glu cultures using correlation analysis indicated that a positive relationship existed between the two measurements. This suggests minimal interference between surfactant expression and chemical component presence in the media, and is in agreement with Robert *et al.* (1989) and Rodrigues *et al.* (2006)'s reports.

Investigation of minimum limit by analysing quantitative surface tension data of the key strains obtained in M9Glu cultures using IDI analysis found the best fit provided by 3-parameter Gamma predicting the minimum to be 24.98 mN/m. The minimum

agreed with the limit predicted in KB* (current study) and existing literature (De Lima *et al.*, 2009, Mohammed *et al.*, 2015, Radzuan *et al.*, 2017, Vilela *et al.*, 2014), thereby suggesting the limit is robust and may not be affected by chemical component presence in the media.

Furthermore, results may suggest the limit is due to bacterial cell wall biology preventing the expression of stronger surfactants. The mechanisms of the limit remain a question. However, limit to bacterial surface activity suggests future surveys of strains with high surface activity may be less promising. Although Mohammed *et al.* (2015) have shown evidence of behavioural variation amongst the low LSTRA strains, it is unclear how much structural and behavioural variation may exist amongst the high performing surfactants. Indeed, differences in behaviour may suggest structural difference (reviewed by Banat *et al.*, 2010).

4.8 Chapter conclusion

In this Chapter, 58 surfactant-expressing pseudomonads strains identified by the drop-collapse assay were tested for surfactant expression using quantitative surface tension measurements by tensiometry. From the 58 strains tested, 46 significantly reduced surface tension of sterile KB* media. A homogenous group of 25 key strains were identified by statistical means with five extra controls for further analysis. Individual Distribution Identification (IDI) analysis was used to determine the

predicted limit for surfactants activity in KB* and M9Glu media, and were found to be in agreement with earlier studies.

Chapter 5

Diversity in surfactant behaviour

Preface

Although surfactants are shown to have different oil-water behaviour, it is unknown whether such differences indicate differing chemical structural composition. In this Chapter, the behaviour of surfactants in different oils (diesel, mineral, vegetable and used lubricating oil) was assessed using different assays and conditions. Hierarchical Cluster Analysis (HCA) and Generalised Linear Modelling (GLM) of the data confirmed diversity amongst surfactants expressed by the key strains. Similarly, some surfactants were seen to have more activity in a particular oil-type, thereby indicating a potential application in biotechnology. The findings in this Chapter show that bioprospecting surfactants by screening only the most active compounds is likely to reveal a range of functionality.

5. 1 Introduction

Pseudomonads produce a range of surfactants with different surface activities and behaviours in air-water (i.e. foams) and oil-water (i.e. emulsions and films) mixtures. In addition, they are complex molecules containing a polar group (hydrophilic) that interacts with water, as well as a non-polar group (hydrophobic tail) that is immiscible in water (reviewed by Seddon *et al.*, 2004). Furthermore, they are characterised by their oil-water behaviour and chemical structural composition (reviewed by Ron and Rosenberg, 2001, Urum and Pekdemir, 2004).

Surfactant behaviour is studied using simple assays that include foam stability, emulsion formation, oil film displacement and haemolytic activity to extract information about surfactant activity and properties (Inès and Dhouha, 2015). Surfactant characterisation using chemical structural analysis has classified surfactants into different classes that include glycolipids, polysaccharides, lipoproteins, lipopeptides or proteins (reviewed by Banat *et al.*, 2010). One of the most widely studied classes of surfactant is cyclic lipopeptides (CLP) produced by different bacterial genera including fluorescent pseudomonads, and these have been found to be diverse in function (Georgiou *et al.*, 1992, Moffitt and Neilan, 2000, Nybroe and Sørensen, 2004, Raaijmakers *et al.*, 2006, reviewed by Ron and Rosenberg, 2010, Ron and Rosenberg, 2001). This class is further divided into groups including surfactin, viscosin, amphisin, tolasin and syringomycin. Furthermore, differences in the structure of CLP and their diversity amongst

producing strains suggest differences in areas of application (de Bruijn and Raaijmakers, 2009a, Moffitt and Neilan, 2000, Olorunleke and Höfte, 2015, reviewed by Ron and Rosenberg, 2010).

The process of identifying surfactants for biotechnology requires a large collection of bacterial isolates to be screened using qualitative and quantitative methods, with these then being further characterised using chemical and structural analysis (reviewed by Biniarz *et al.*, 2016, Mohammed *et al.*, 2015). However, this process is expensive, time-consuming and requires sophisticated equipment. It is therefore impractical to characterise an extensive collection of surfactants to choose from for future prospecting using this technique. A viable alternative is to characterise surfactants using simple behavioural assays including emulsion formation, form stability and oil displacement. This would obtain evidence of chemical and structural diversity that would enable the selection of novel surfactants for future study or biotechnological testing.

5.2 Research objectives

This Chapter reports on investigating diversity amongst surfactants expressed by key pseudomonad strains producing a limited range of very low surface tension in modified King's B (KB*) broth culture (24 – 26 mN/m) using their behaviour. The

results obtained could be of high significance in selecting strains for future chemical analysis and biotechnological testing.

The objectives of this research are to:

I. Determine the behaviour of surfactants produced by the key strains using emulsion, foam stability and oil-displacement assays;

II. Investigate the effect of buffer chemistry (pH and salt) on surfactant behaviour;

III. Investigate surfactant chemical and structural diversity from simple behaviour assays data (i and ii above) using HCA (Hierarchical Cluster Analyses) and a General Linear Model (GLM);

IV. Assess the behaviour of key surfactants after semi-purification using quantitative tensiometry in a range of pH and salt concentrations.

5.3 Determination of oil-water behaviour of surfactants produced by key strains

The work presented in Chapter 4 resulted in a collection of key strains with very low surface tension value and controls. Initial phenotypic characterisation in Chapter **3** showed that the strains were diverse in phenotypic characteristics. Moreover, 16S rDNA and Analytical Profiling Kit identified these strains as pseudomonads.

In order to assess the behaviour of surfactants expressed by the key strains (25 low-LSTRA), a range of simple behaviour assays including emulsion indices, foam stability and oil displacement were employed reflective of work by Reiling *et al.* (1986), Cooper and Goldenberg (1986) and Morikawa *et al.* (1993).

5.3.1 Emulsion assay

In the emulsion assay, the degree to which surfactants expressed by the key strains mixed with oil and water to form an emulsion was investigated. This behaviour was assessed using diesel oil, as described in Section **2.12**. The relative aqueous (A_I), emulsion (E_i) and oil (O_i) indices showed that surfactants produce a diverse response in oil-water mixtures ($p < 0.0001$). Moreover, surfactants may show a high emulsion index (E_i) (for example, strains 1, 6 and 21), if surfactants have influenced oil movement into the emulsion phase or if a more aqueous phase was absorbed into the emulsion phase. Similarly, a low oil index (O_i) is the result of more oil

moving into the emulsion phase, and in fact these scenarios occurred when a low aqueous index (A_i) was obtained, as shown in Figures **5.1.**

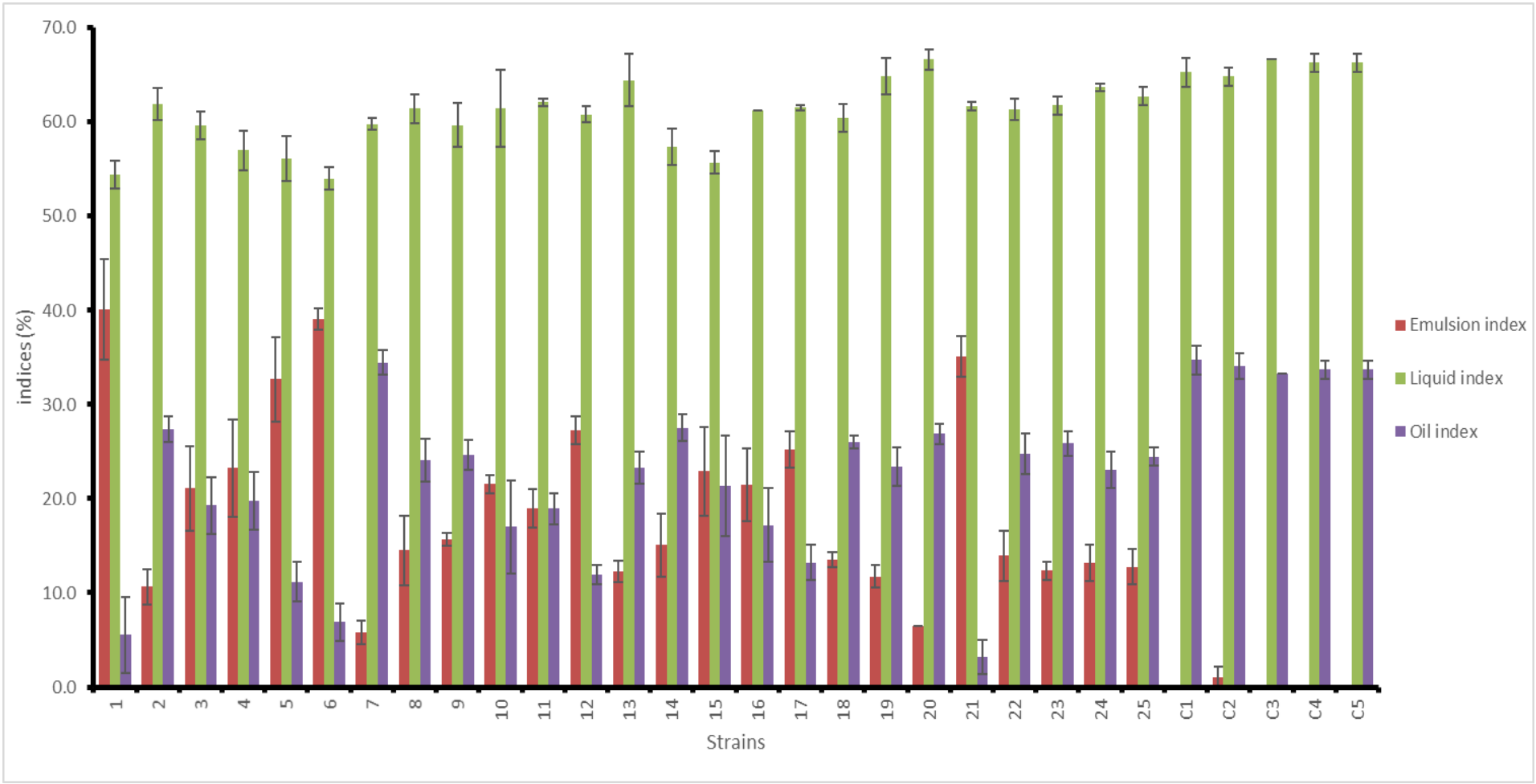
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
indices (%)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
C1
C2
C3
C4
C5
Strains
Emulsion index
Liquid index
Oil index

Figure 5.1: Diversity among *Pseudomonas* spp. strains with low surface tension measurements on emulsification assay. Emulsification assays were conducted using protocol stated in Chapter 2 by mixing 18 h KB* cultures, deionised water and diesel. The corresponding emulsion, liquid and oil indices were measured after 24 hrs inclubation period with a mean ± SE (n = 3). The results showed there is a significant difference among the mean values for the key strains on Emulsion index with red bars (ANOVA $F_{29, 60}$ = 19.247; P < 0.0001); in Liquid inex with green bars (ANOVA $F_{29, 60}$ = 38.217; P < 0.0001) and in Oil index with blue bars (ANOVA $F_{29, 60}$ = 14.276; P < 0.0001). However, the control strains resulted in zero value in emulsion index.

5.3.2 Foam stabilisation

In order to assess surfactants expressed by the key strains for foam stabilisation, a modification of the protocol reported by Reiling *et al.* (1986) was utilised. The percentage foam height reduction showed that surfactants differ significantly in their ability to stabilise foam ($p < 0.0001$). Moreover, surfactants expressed by strains one, four and 21 showed high foam stability compared to other strains (Figure **5.2**). However, an unexpected foam stabilisation was observed in one of the control strains (c5) which could have been as a result of the strain producing another active compound that influenced foam stability behaviour. Other control strains completely drained foam after two hours of the assay.

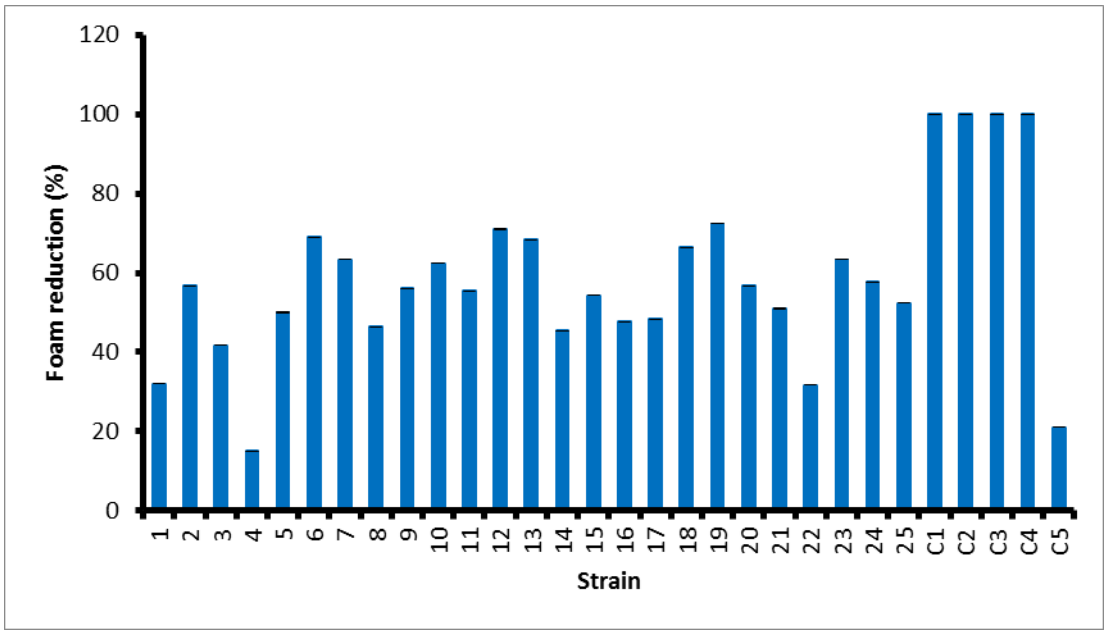

Figure 5.2 Diversity among *Pseudomonas* spp. strains with low surface tension measurements on stabilisation of foams. Foam stability was assessed using replicate 18 h KB* cultures with a mean ± SE (n = 3) measurement noted. There is a significant difference among the mean values for the key strains (ANOVA $F_{29, 60}$ = 24.680; P < 0.0001). 100% foam reduction resulted in no foam.

5.3.3 Summary

The behaviour of surfactants expressed by the key strains was investigated using emulsion indices and foam stabilisation assays. Results revealed that a significant variation exists among surfactants expressed by the key strains.

5.4 Effect of buffer chemistry (pH and salt) on surfactant behaviour

In order to investigate the effect of buffer chemistry on surfactant behaviour, 12 independent oil displacement assays using 50 mM Tris-HCl buffer (pH 8), deionised water (pH 6) and 200 mM NaCl solution were used as the aqueous phases. While one of the four oils (diesel, mineral, used engine oil and vegetable oil) was used as a thin top oil layer to study the difference in surfactant behaviour under the different conditions due to possible differences in surfactant chemical structure. In these assays, the diameter of the cleared zone (displaced zone) in each case was measured after adding an aliquot of surfactant to the centre of the thin oil layer.

The surfactants expressed by the key strains significantly responded differently to each of the 12 independent oil displacement assays (see Appendix **A5.1** for *p*-values table), thereby suggesting that pH and salt influence surfactant activity (Figures **5.3** - **5.6**). This result was in support of the previous emulsion indices and foam stabilisation assay in which differences were observed among oil-water and air-water behaviour of surfactants expressed by the key strains. This suggests differences in chemical and structural composition.

The results generated from the 12 independent oil displacement assays, emulsion indices and foam stability assay were further examined in the following sections of this Chapter.

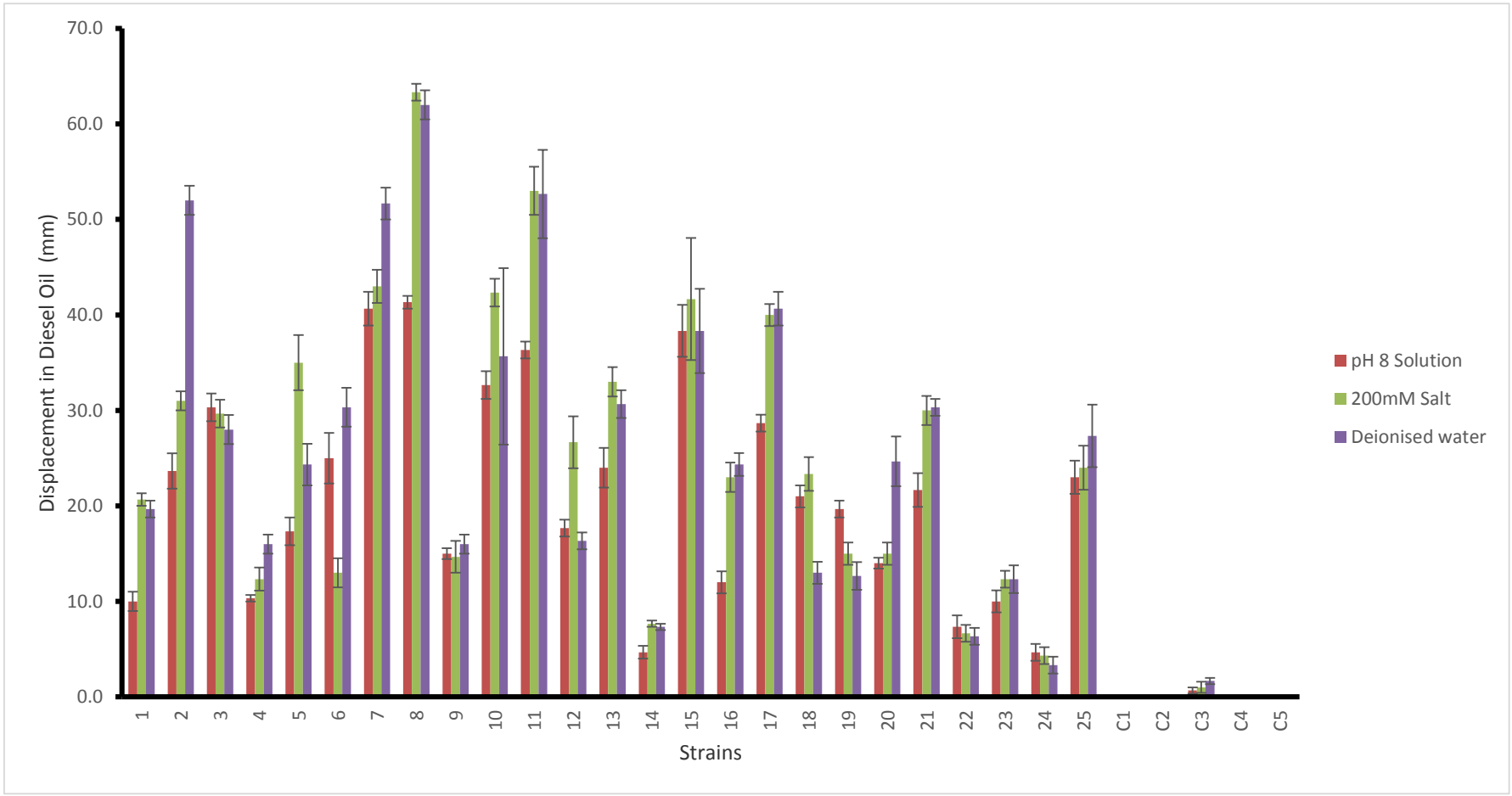
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Displacement in Diesel Oil (mm)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
C1
C2
C3
C4
C5
Strains
pH 8 Solution
200mM Salt
Deionised water

Figure 5.3 Diversity of surfactants expressed by *Pseudomonas* spp. with low surface tension activity upon diesel oil displacement under the influence of pH and salt. Oil displacement was assessed using 18 h KB* cultures with either pH 8 solution (red), 200mM of salt solution (green) or deionised water (purple) overlaid with diesel oil. Measurements of diameter are noted mean ± SE (n = 3). The results indicates there is a significant difference among the mean values for the entire key in pH 8 (ANOVA $F_{29, 60}$ = 98.845; $P < 0.0001$); 200mM salt (ANOVA $F_{29, 60}$ = 85.001; $P < 0.0001$) and in deionised water (ANOVA $F_{29, 60}$ = 51.116; $P < 0.0001$). However, four control strains have a value of zero.

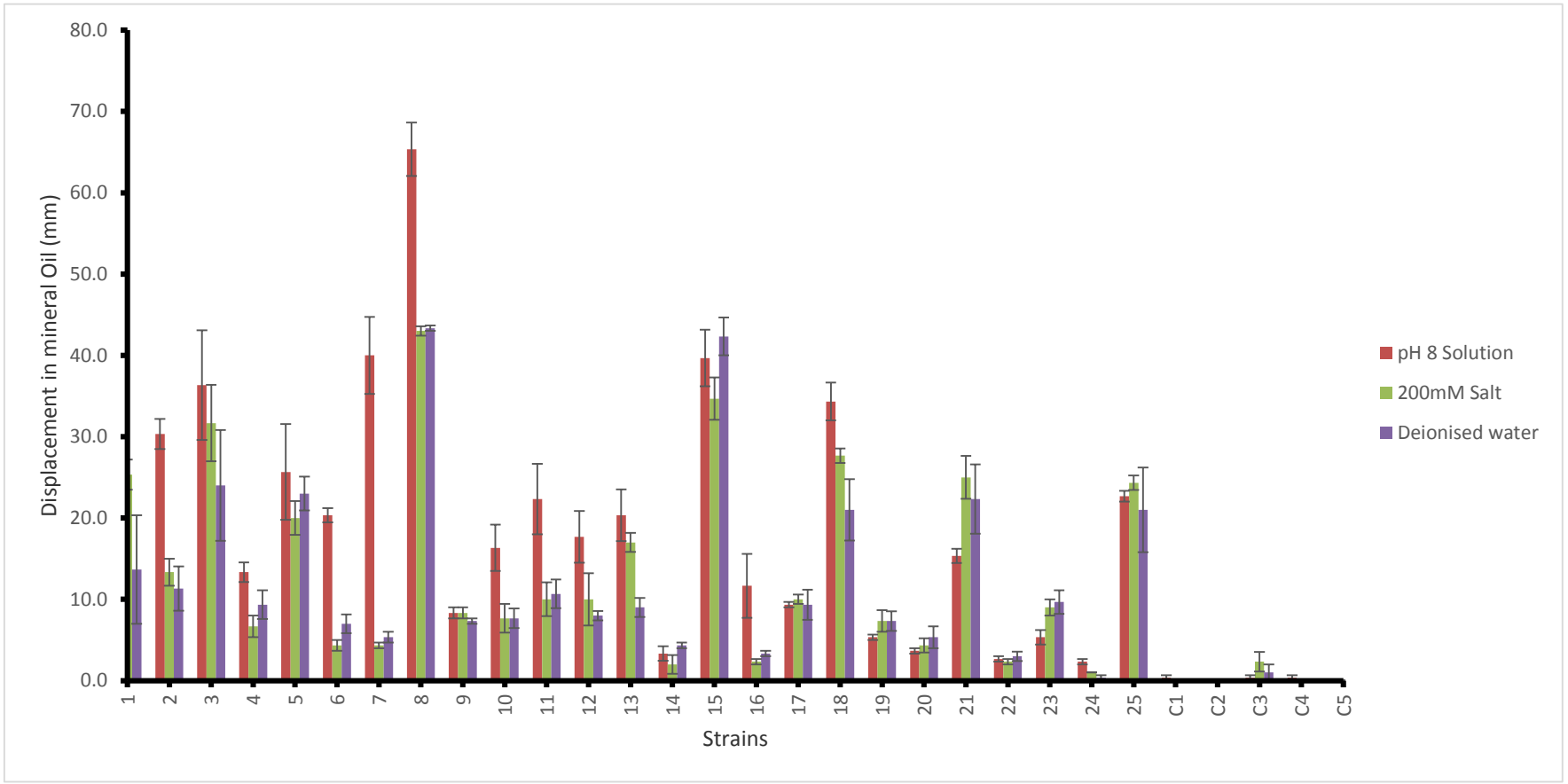
Displacement in mineral Oil (mm)
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
C1
C2
C3
C4
C5
Strains
pH 8 Solution
200mM Salt
Deionised water

Figure 5.4 Diversity of surfactants expressed by *Pseudomonas* spp. with low surface tension activity upon mineral oil displacement under the influence of pH and salt. Oil displacement was assessed using 18 h KB* cultures with either pH 8 solution (red), 200mM of salt solution (green) or deionised water (purple) overlaid with mineral oil. Measurements of diameter are noted mean ± SE (n = 3). The results indicates there is a significant difference among the mean values for the entire key in pH 8 (ANOVA $F_{29, 60}$ = 35.571; P < 0.0001); 200mM salt strains (ANOVA $F_{29, 60}$ = 54.367; P < 0.0001) and in deionised water strains (ANOVA $F_{29, 60}$ = 20.116; P < 0.0001). However, four control strains have a value of zero.

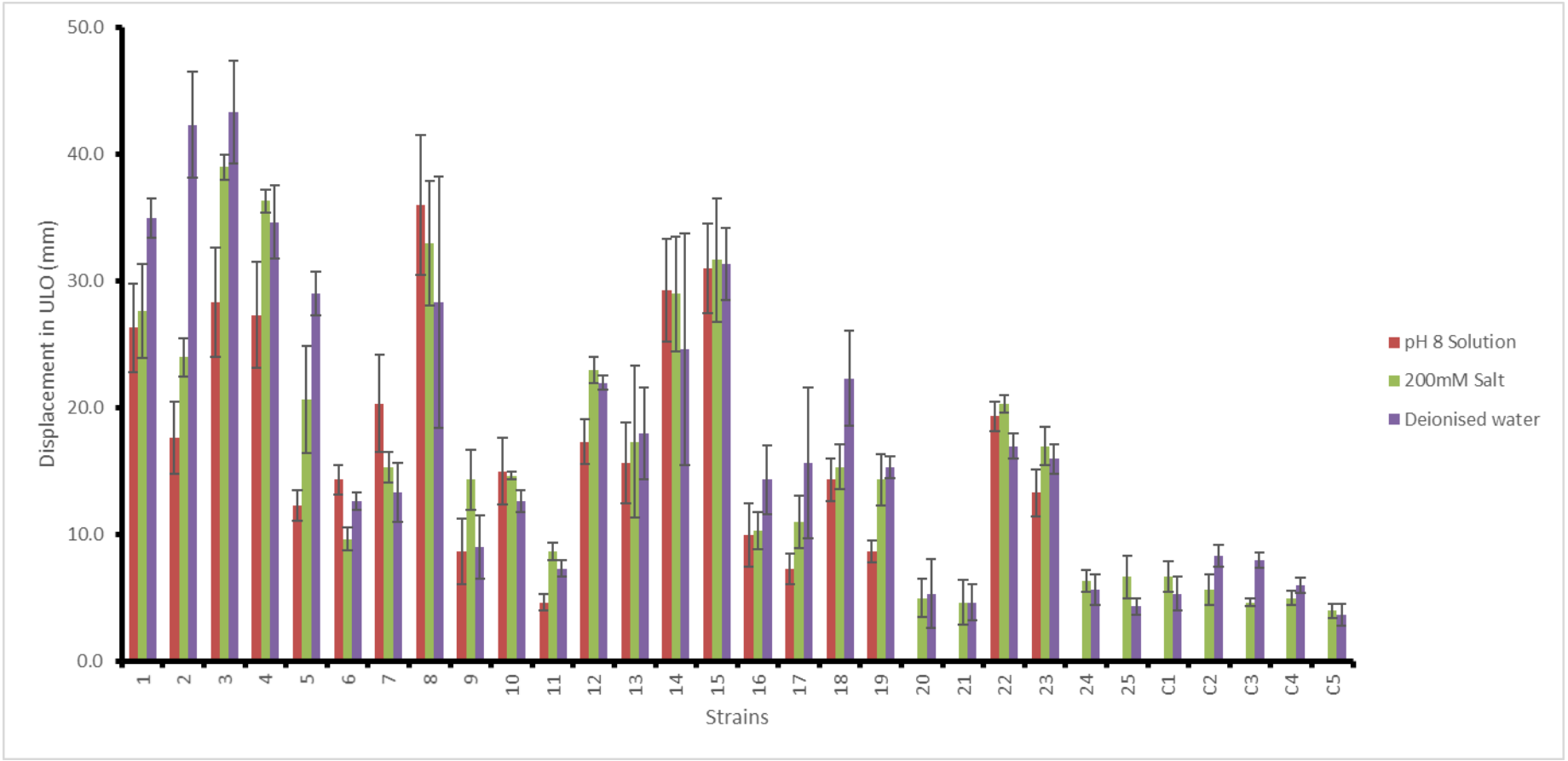
50.0
40.0
30.0
20.0
10.0
0.0
Displacement in ULO (mm)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
C1
C2
C3
C4
C5
Strains
pH 8 Solution
200mM Salt
Deionised water

Figure 5.5 Diversity of surfactants expressed by *Pseudomonas* spp. with low surface tension activity upon used lubricating oil displacement under the influence of pH and salt. Oil displacement was assessed using 18 h KB* cultures with either pH 8 solution (red), 200mM of salt solution (green) or deionised water (purple) overlaid with ULO. Measurements of diameter are noted mean ± SE (n = 3). The results indicates there is a significant difference among the mean values for the entire key in pH 8 (ANOVA $F_{29, 60}$ = 20.770; P < 0.0001); 200mM salt strains (ANOVA $F_{29, 60}$ = 17.946; P < 0.0001) and in deionised water strains (ANOVA $F_{29, 60}$ = 12.044; P < 0.0001). However, four control strains have a value of zero.

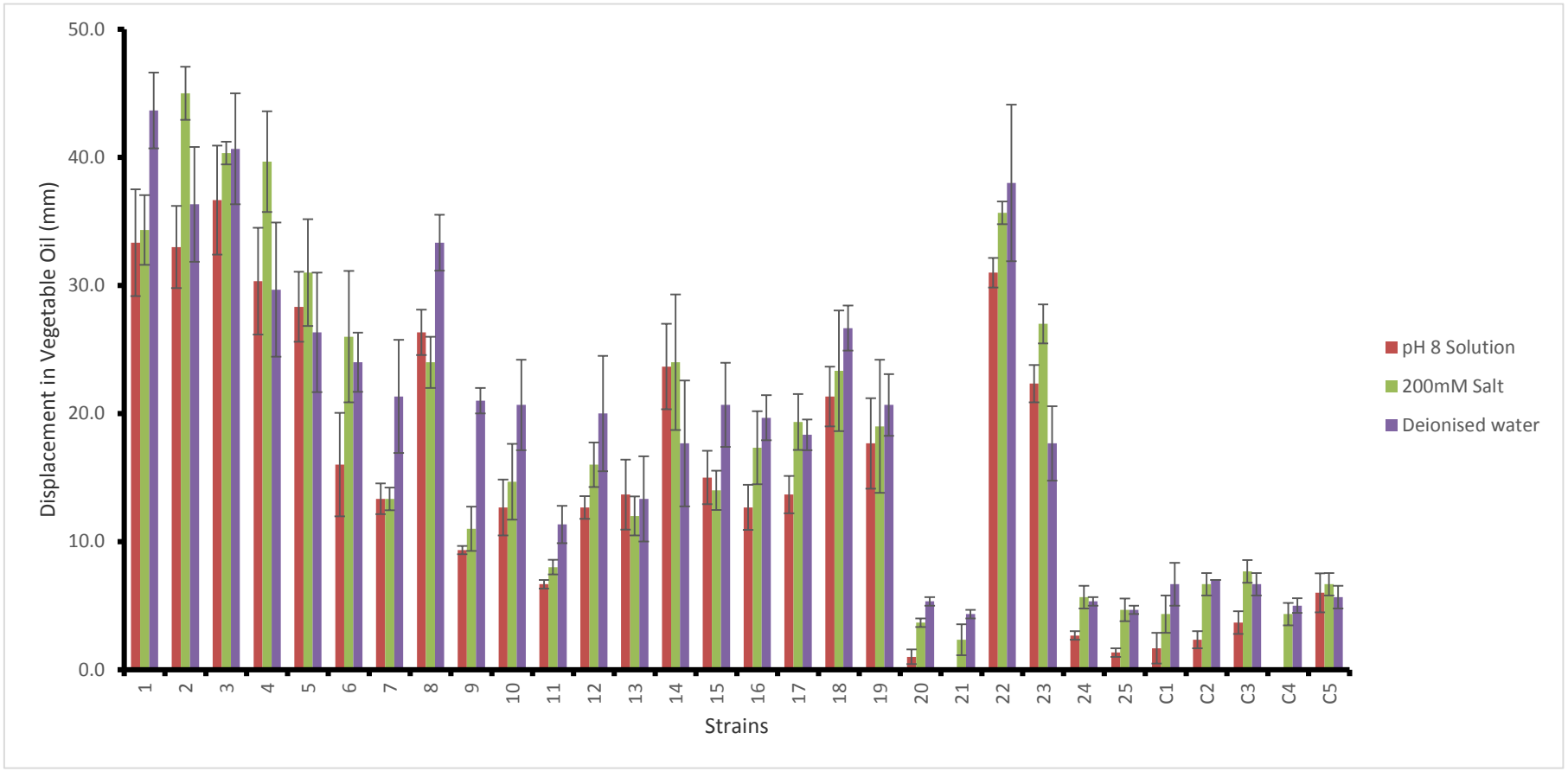
50.0
40.0
30.0
20.0
10.0
0.0
Displacement in Vegetable Oil (mm)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
C1
C2
C3
C4
C5
Strains
pH 8 Solution
200mM Salt
Deionised water

Figure 5.6 Diversity of surfactants expressed by *Pseudomonas* spp. with low surface tension activity upon vegetable oil displacement under the influence of pH and salt. Oil displacement was assessed using 18 h KB* cultures with either pH 8 solution (red), 200mM of salt solution (green) or deionised water (purple) overlaid with vegetable oil. Measurements of diameter are noted as Mean ± SE (n = 3). The results indicates there is a significant difference among the mean values for the entire key in pH 8 (ANOVA $F_{29, 60}$ = 25.520; $P < 0.0001$); 200mM salt strains strains (ANOVA $F_{29, 60}$ = 23.287; $P < 0.0001$) and in deionised water strains (ANOVA $F_{29, 60}$ = 14.896; $P < 0.0001$). However, four control strains have a value of zero.

5.4.1 Section summary

Investigating the effect of pH and salt on surfactant behaviour demonstrated a significant variation among surfactants expressed by the key strains in each of the 12 independent oil displacements.

5.5 Diversity in surfactant behaviour

The work in the previous sections reveals that key surfactants differ significantly in each of the air-water and oil-water behaviour assays. In this section, information generated from these simple behaviour assays was used to investigate the chemical and structural diversity amongst surfactants expressed by the key strains. A multivariate method of analysis (Hierarchical Cluster Analysis (HCA) was used. HCA output is in the form of star-burst like constellation dendrograms from which clusters of similar strains can be traced. Moreover, HCA can be useful when investigating the similarities between assay data by two-way clustering, thereby allowing selection of the best assays that differentiate amongst strains.

To assess surfactant behavioural diversity, data generated from the behaviour assays including the foam stability, emulsion indices and the 12 independent oil displacement assays were analysed using the multivariate method of analysis, and visualised using the constellation dendrogram. HCA divided surfactants expressed by the strains into six major groups of 2 - 7 strains based on similarities in surfactant behaviour (Figure **5.7**). The two largest groups (groups D and F) contained seven strains each. However, group F contained all five controls (c1 – c5) which confirmed similarity among the control strains. The remaining 23 strains were divided into five groups, which perhaps implies the possibility of five major different types of surfactant with differing chemical and structural composition.

The constellation dendrogram produced in this research using data from simple behavioural assays provided a useful tool for choosing surfactants for future testing. For instance, research with time, resource or other constraints could not examine a large collection of surfactants, so selection of strains 4, 5, 15, 23, 25 and c1 could be utilised. These strains provide representation of the full chemical diversity among surfactants expressed by the key strains. Alternatively, selection of strains 4 and 14 means selecting surfactants with a degree of similarity in surfactant chemical structure. However, minimal structural differences between 4 and 14 would be expected when compared to 10 and 14, as they may have greater structural variation.

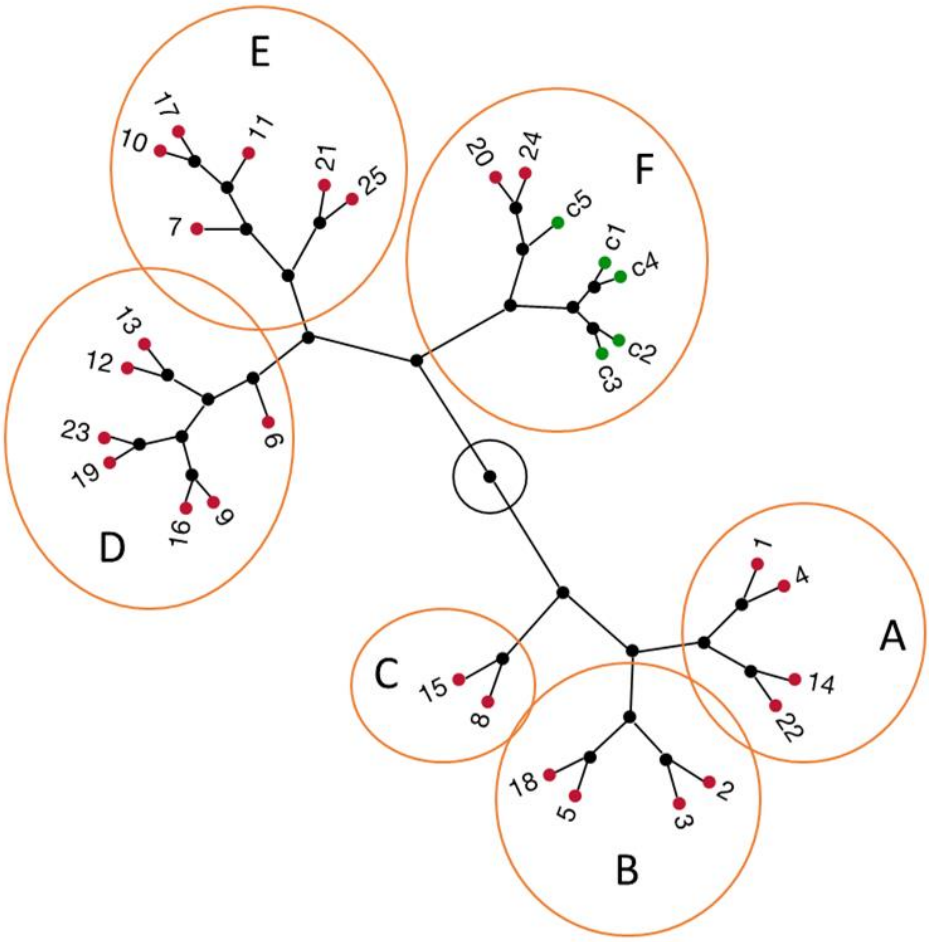

Figure 5.7 Hierarchical cluster analysis groups key strains based on oil-water behaviour. The data obtained from simple behaviour assays including the foam stability, emulsion indices and 12 independent oil displacement assays using 50 mM Tris-HCl buffer (pH 8), deionised water (pH 6) and 200 mM NaCl solution as the aqueous phase with one of the four oils (diesel, mineral, used engine oil and vegetable oil) as the thin top oil layer was assessed for diversity using the multivariate cluster method of analysis. This figure shows a constellation dendrogram in which similar surfactants are placed on nearby branches (rooted at the Open Circle). Red indicates surfactant-expressing strains while green indicates control strains. The HCA was generated using word method

Similarly, a two-way cluster analysis of a larger dataset was conducted to investigate surfactant response to behaviour assay. In this analysis, the behaviour assay data, including the emulsion indices, foam stability and the 12 independent oil displacement assays with 50 mM Tris-HCl buffer; pH 8), deionised water (pH 6) and 200 mM NaCl solution) topped with one of the four oils (diesel, mineral, used engine oil and vegetable oil) as the thin top oil layer, were used to produce a two-way cluster dendrogram. The groupings were based on the surfactant behaviour assays and buffer conditions. The latter divided the strains into six previously identified groups, while grouping based on behaviour showed more diversity amongst oils type than between conditions (pH and salt). It is noteworthy that surfactants differed more significantly in their interaction with different oils than with the buffer. This indicates their potential application in various areas of biotechnology (Figure **5.8**). It was observed that surfactant behaviour in mineral oil plus conditions was more related to behaviour in diesel than used lubricating oil (ULO). In addition, it is noteworthy that the foam stability assay separated the four oil types.

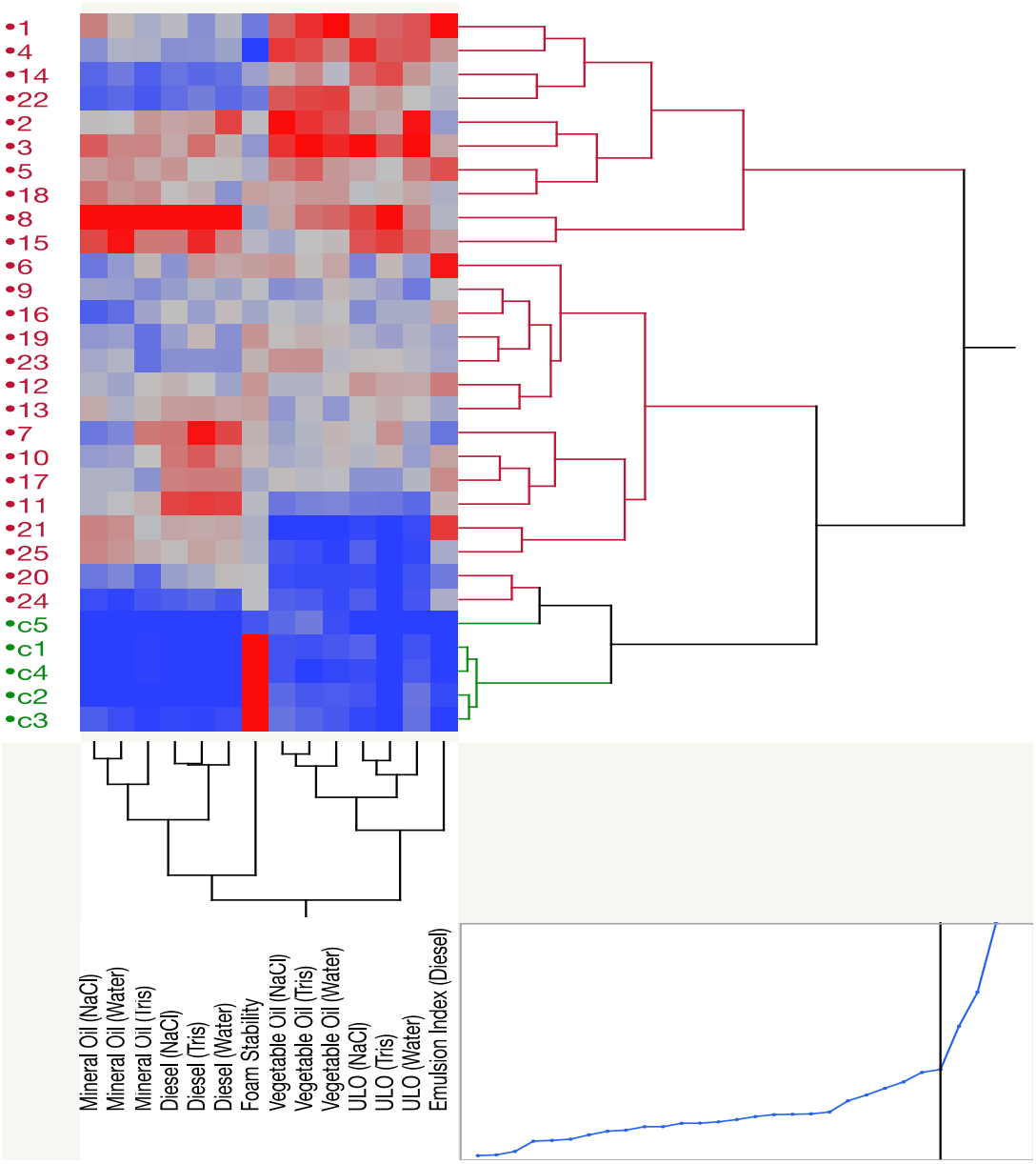

Figure 5.8 Behavioural diversity existing among surfactants expressed by the key strains. A two-way cluster dendrogram was used to assess diversity among surfactants expressed by the key strains using data generated from a simple behaviour assay. This is a two-way grouping based on surfactant behaviour assays in the column, and groupings based on the condition (pH and salt) in the rows. The colours indicate diversity: blue shows more similarity and red signifies differences. Surfactants show higher diversity among oil types than with conditions (salt and pH). The scree plot at the bottom shows several factors are responsible for the variability in surfactant behaviour, with four factors being notable.

5.5.1 Section summary

Investigating surfactant diversity using HCA resulted in the grouping of surfactants into six major groups based on their oil-water behaviour data. This indicates a major chemical and structural diversity among surfactants expressed by the key strains. Moreover, a two-way cluster dendrogram shows more diversity in the way surfactants associate with oils than with the buffer conditions, thereby indicating potential application in different areas of biotechnology.

5.6 Modelling behaviour of surfactants expressed by the key strains

To further understand the relationship among surfactants expressed by the key strains, surfactant oil-water behaviour data was modelled using the General Linear Model approach (GLM). GLM is a powerful tool for developing models that contain multiple variables. The model is designed to study fitness of data and to examine variance among the multivariate behaviour dataset in order to reveal the effect of the strains, oil type and buffer condition on surfactant behaviour. The advantage of GLM is that each of the important variables including the condition (50 mM Tris-HCl buffer, pH 8); deionised water, pH 6 and 200 mM NaCl solution), oil types (diesel, mineral, used engine oil and vegetable oil) and strains are modelled independently and do not have to be pooled into a single complex factor.

In this model, the mean data of the 12 independent oil displacements were modelled with displacement as the variable, while the oil types, condition and strains were treated as the source of effects. The fitness test showed that the data fitted the model well. This was indicated by a high goodness of fit value (R^2 = 0.894). Futhermore, the model analysis of variance (ANOVA) demonstrated that there was a significant difference among all the predictive variables (ANOVA $F_{220, 858}$ = 31.376; P < 0.0001). These indicate the model was robust and that the assays were credible and well-implemented.

The effect test revealed that buffer conditions (50 mM Tris-HCl (pH 8), deionised water (pH 6) and 200 mM NaCl solution), oil types (diesel, mineral, used engine oil and vegetable oil) and strains had a significant impact on surfactant behaviour (see Table **5.1** for *p*-values). This corresponds to the ANOVA earlier described in Section **5.4** and the HCA in Section **5.5**. It is not surprising, however, that replicates also significantly impacted on the model (Table **5.1**) because the cleared zone was not always a perfect circle, and therefore some variation was naturally introduced into this measurement.

Table 5.1: Summary of *p*-values obtained from the effect test examined using the General Liner Model (GLM).

Source	DF	SSQ	F Ratio	*P*-value
Model	220	181853.73	31.36	<0.0001
Condition	2	884.06	16.78	<0.0001
Strain * oil	87	58560.80	25.55	<0.0001
Oil * condition	6	3696.29	23.38	<0.0001
Oil	3	8795.23	111.28	<0.0001
Strains	29	106126.56	138.90	<0.0001
Conditions * strains	58	2797.49	1.83	0.0002
Replicate	1	157.15	5.97	0.0148

DF = Degree of freedom
SSQ = Sum of squares

5.6.1 Section summary

Modelling surfactant behaviour data using GLM revealed that the key strains produce different surfactants that exhibit different behaviours which could be influenced by the buffer condition (pH, salt) and oil type. This model strongly supports the earlier assumption made by the HCA and thereby indicates significant diversity in chemical and structural composition of surfactants expressed by the key strains.

5.7 Investigating the effect of media and other growth components on surfactant behaviour

The HCA discussed in section **5.5** classified surfactants into five major groups and suggested limited structural variation among surfactants expressed by the closely related *Pseudomonas spp.* strains (strains that share the same group). It is unclear as to whether media or other growth components contributed to the differences observed among close and far-related surfactant groups identified in Figure **5.7**. Consequently, strains 1-5, 10, 15, 20 and 25 were selected to ascertain if media and other growth components contributed to the surfactant behaviour observed, and if this could explain the difference observed among surfactants sharing a similar group.

Surfactants expressed by the selected strains were semi-purified using the protocol reported by Song *et al.* (2015). The semi-purified surfactants were re-suspended in different pH and NaCl salt concentrations (200, 400, 600, 800 and 1000 mM), and different pH conditions (4, 6, 8, 10 and 12) before quantitative surface tension measurement.

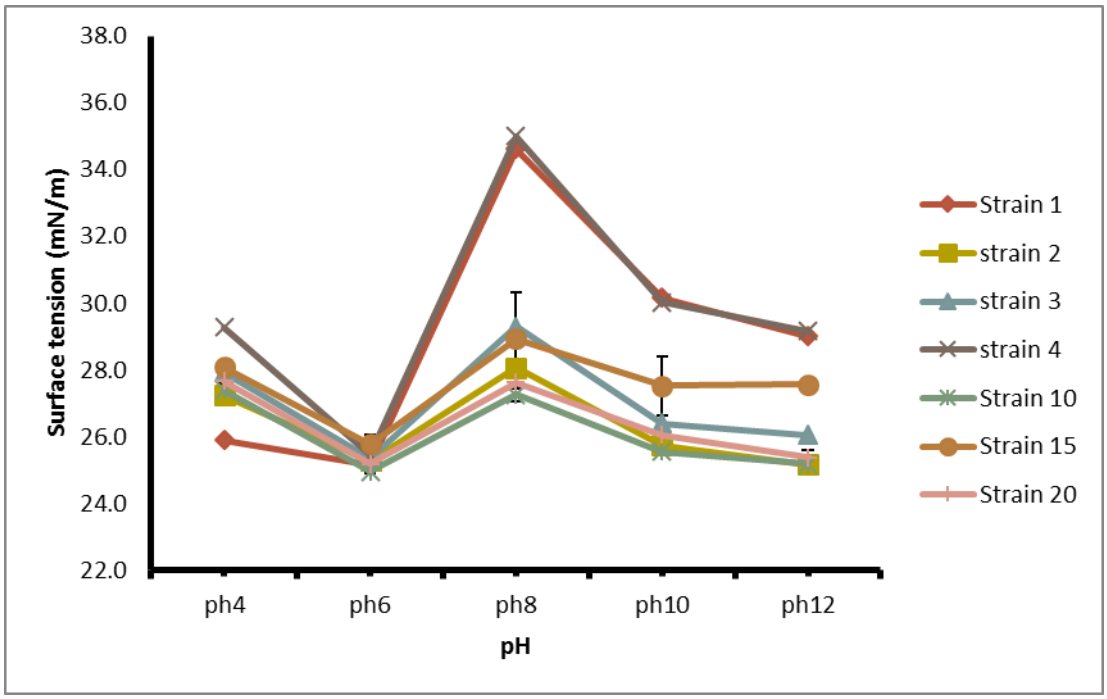

Figure 5.9 Surfactant activity affected by changes in pH. The effect of pH on surfactant activity was tested by measuring surface tension of the semi-purified material in different pH ranges. A mean ± SE (n = 3) of surface tension measurements is shown. There is a significant difference among surfactant activity at different pH levels (ANOVA $F_{30, 42} = 26.943$; $P < 0.0001$).

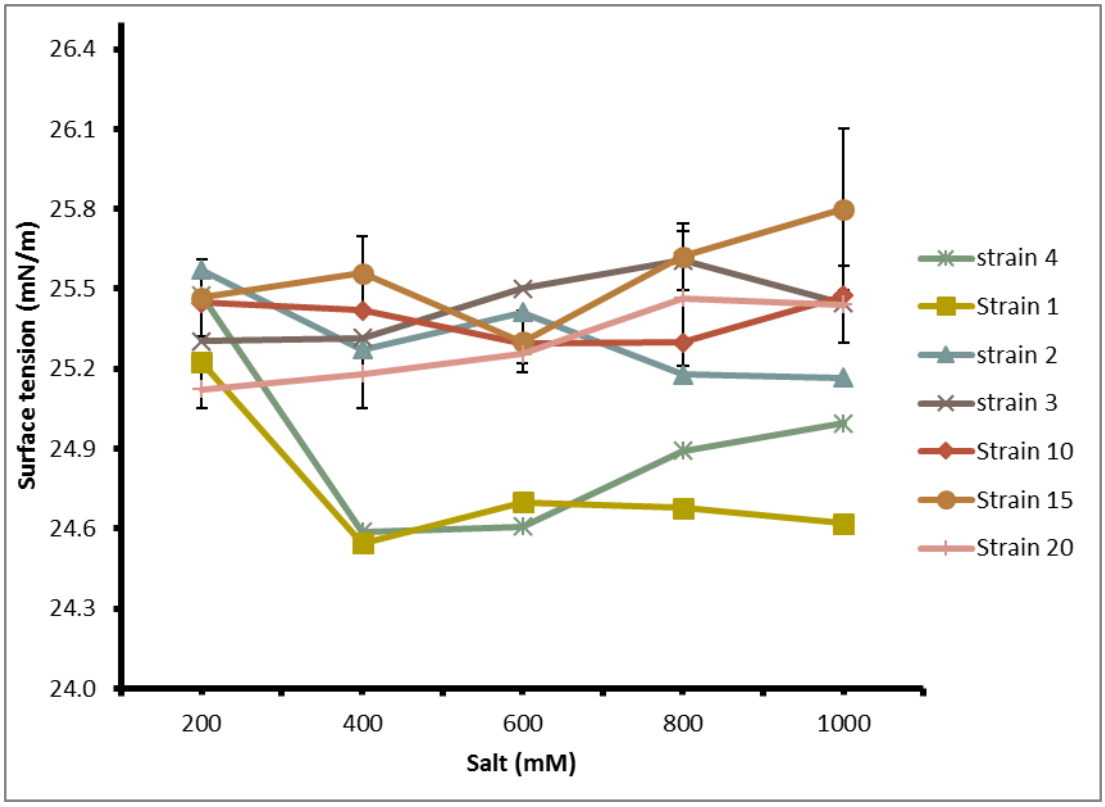

Figure 5.10 Surfactant activity affected by salt concentration. The effect of salt concentration on surfactant activity was assessed by measuring the surface tension of the semi-purified material in different salt concentrations. A mean ± SE (n = 3) of surface tension measurements is shown. There is a significant difference among surfactant activity at various salt concentrations (ANOVA $F_{30, 42} = 3.467$; $P < 0.0001$).

Analysis of variance among surface tension measurements in both different NaCl salt concentrations and pH conditions revealed that a significant difference existed among surfactants expressed by the selected strains ($p < 0.0001$). It was observed that surfactant expressed by strains 1 and 4 that appeared to be relatively close in the HCA classification, yet differed at pH 4 and different salt concentrations. Similarly, far-away strains on HCA, for instance strains 1 and 20, exhibited different surface activity at almost all pH and salt concentrations (Figure **5.9** and **5.10**). Moreover, the selected strains showed various surface activity particularly at pH 8 and various salt concentrations. This indicates media or other growth components have little or no influence on surfactant behaviour. This also confirms the diversity earlier observed in previously presented HCA in Section **5.5**, as well as the differences in chemical and structural composition of surfactants. However, surfactants expressed by strains 5 and 20 could not be extracted using the adopted protocol, which suggests that the strains could be expressing an entirely different class of surfactants.

5.7.1 Section summary

Investigating the effect of media and other growth compounds on surfactant behaviour by testing the semi-purified surfactants from representative strains confirmed that there was a significant behavioural variation among surfactants expressed by the key strains, with no interference by the media or other growth components.

5.8 Chapter discussion

In this Chapter, surfactants expressed by the key strains with strong surface activity (24 – 26 mN/m) were investigated for chemical and structural variation using simple behaviour assays including foaming, emulsion and oil displacement assays.

Literature, including Burch *et al.* (2010); Bouchez *et al.* (1999); Mandal *et al.* (2013); Maier *et al.* (2003); Raaijmakers *et al.* (2010); Roongsawang *et al.* (2010); Tran *et al.* (2008) and Youssef *et al.* (2004), has reported on surfactant behaviour using simple oil behaviour assays including emulsion, foam stability, and oil displacement assays. Although these studies may involve a single or a few strains, differences in surfactant behaviour may perhaps suggest chemical and structural differences (Haferburg *et al.*, 1986, Makkar and Rockne, 2003, Sánchez *et al.*, 2007)**.**

In this Chapter, the key strains were studied using different oil-water behaviour assays including emulsion indices, foam stability and 12 independent oil-displacement assays using diesel, mineral, ULO and vegetable oils, as well as three different buffer conditions (distilled water, pH 6.0; 200 mM NaCl; and 50 mM Tris, pH 8.0) to assess diversity in surfactant chemical and structural composition. Initial analysis of variance indicated a significant variation exists among surfactants in each of the behaviour assays, and this conforms to findings in existing literature (reviewed by Lin, 1996, Morikawa *et al.*, 2000, Shin *et al.*, 2008).

Investigating surfactant behaviour for chemical diversity using the HCA (Hierarchical Cluster Analysis) divides surfactants expressing strains into six groups, thereby indicating six major chemical and structural differences. Critically, the surfactant behaviour dendrogram resulted in more groups than could be explained by differences in culture surface tensions examined earlier by ANOVA and Tukey-Kramer HSD in Chapter **4.** This means the key strains are expressing structurally different surfactants, even though they have similar surface activity strength.

The two-way cluster analysis of surfactant behaviour showed more diversity among oil types (diesel, mineral, used lubricating oil and vegetable oil) than the buffering condition used (distilled water, pH 6.0; 200 mM NaCl; and 50 mM Tris, pH 8.0). This perhaps indicates the prospect of surfactants within different areas of biotechnology. For instance, the surfactant expressed by strain 22 may be better utilised in the food industry than in bioremediation due to its high activity in vegetable oil when compared to ULO. Surfactants with a high affinity to mineral oil and used lubricating oil could potentially be useful in the cosmetic and bioremediation industries. It is interesting to note that surfactant behaviour in oils and under varying conditions suggests that usefulness of surfactants may vary depending upon the area of application. These findings are in support of studies by Dadrasnia and Ismail (2015); Jones (1998) and Lawniczak *et al.* (2013) that report on the potential application of surfactants in bioremediation. Other reports include their possible application in the cosmetic industry (Morita *et al.*, 2009, Nguyen *et*

al., 2010), in the food industry (Nitschke and Costa, 2007, Shepherd *et al.*, 1995) and in biomedical science (Das *et al.*, 2008, Kitamoto *et al.*, 2002, Singh and Cameotra, 2004).

The HCA grouping was supported by the GLM in which the effect test confirmed diversity among surfactants expressed by the key strains in both oil and buffer conditions used (pH and salts). It is important to note that this model is robust as indicated by the high goodness of fit value, thereby indicating that data is credible and all the assays are well conducted. GLM modelling is a powerful tool for assessing multivariate data sets and could be useful when dealing with data that have more than one predictor for a set of variables (Lepš and Šmilauer, 2003).

Investigating the effect of media and other growth components on surfactant diversity using semi-purified surfactants in a range of different pH and salt concentrations showed no effect on the diversity observed. This result further confirmed the slight differences observed in HCA. These findings unanimously agree with the results from checking the effect of media and growth components on surfactant activity, with the minimum prediction in Chapter **4** and with existing literature on the effect of salt and pH on surfactant activity (Andrade Silva *et al.*, 2014, Robert *et al.*, 1989, Tabatabaee *et al.*, 2005, Zhang and Miller, 1992)**.**

5.9 Chapter conclusion

The work undertaken in this Chapter demonstrates significant behavioural variation within a collection of high-performing surfactants expressed by the key strains. This means bioprospecting for new bacterial surfactants, by screening for only the most surface-active compounds, is likely to reveal a range of surfactants having different behaviours in air-water and water-oil mixtures.

The constellation dendrogram produced in this Chapter using data from simple behavioural assays provides a useful tool for identifying surfactants for future testing.

Chapter 6

Discussion

Preface

Surfactants have important natural and industrial roles. They are identified using qualitative and quantitative assessments, and are often further characterised by chemical structural analysis and molecular based techniques. The later techniques are expensive and require sophisticated equipment. In this thesis, surfactant-expressing pseudomonads were recovered from soil using pseudomonad selection agar. The strains were first screened for surfactant expression using a qualitative technique, before subjecting them to quantitative tensiometry. A set of 25 best-performing strains were selected (key strains) and characterised using growth-based assays and molecular techniques (Chapters 3 and 4). This was prior to investigating the isolates for chemical structure and function diversity using a range of surfactant behavioural assays (Chapter 5).

6.1 Introduction

Bacteria are widely distributed in all environments (air, soil and water). They are diverse and exhibit different lifestyles (Pierret *et al.*, 2007, Raaijmakers *et al.*, 2009). Soil, rhizosphere in particular, is a complex ecosystem that is a hotspot for microbes that are fertile, easily reachable and a safe source for the isolation of bacteria (Hinsinger and Courchesne, 2008, Hinsinger and Marschner, 2006). Surfactant-expressing pseudomonads are rapidly isolated from both contaminated and uncontaminated environments and are shown to have diverse functions (Ruggeri *et al.*, 2009). Over the years, surveys for surfactant-expressing bacteria have been achieved using qualitative and quantitative assays, with few studies characterising three or more surfactants using chemical structural analysis. This could be due to the expensive nature of the process and possibly the large number of samples involved (Heyd *et al.*, 2008, Liu *et al.*, 2012). Surfactants' chemical structural diversity could be of importance to biotechnology, and so an approach is needed to guide selection of surfactant-expressing strains for future biotechnological testing.

In bioprospecting studies, surfactants that reduce the surface tension the most are generally selected for testing in biotechnology. Consequently, a number of studies have reported strong surfactant activity ranging between 24-30 mN/m. However, it is unclear how much structural and behavioural variation exists amongst these high-performing compounds. In this thesis, the range of surfactant behaviours produced by *Pseudomonas* spp. was investigated, with the long-term goal being the identification of novel surfactants with biotechnological application in

bioremediation, food and medical biotechnology (e.g. the identification of a surfactant usable within single or multiple applications).

6.2 Isolation and characterisation of surfactant producing *Pseudomonas* spp. from soil

Studies have shown that surfactant-expressing bacteria can be isolated from different environments, including contaminated and uncontaminated sites (reviewed by Maier, 2003). This is done either by enrichment of cultures or a qualitative assessment such as the drop-collapse test to select surfactant-expressing strains (Eddouaouda *et al.*, 2012, Xia *et al.*, 2012). However, two major bacterial genera, *Bacillus* and pseudomonads, are commonly known to express surfactants. These therefore could be a potential target for bioprospecting survey studies, in which novel surfactants with high activity and behaviour are of interest (Burch *et al.*, 2010, reviewed by Raaijmakers *et al.*, 2010, Weyens *et al.*, 2009).

In this thesis, a diverse bacterial collection of 251 pseudomonads strains were isolated from Dundee Botanic Garden soil using PSA+CFC (Table **3.1**). The isolates were first screened for liquid surface tension-reducing ability (LSTRA) by a drop-collapse assay of 18 h KB* broth cultures. Of the total isolates, 58 strains were positive LSTRA. Although this study isolated strains from uncontaminated sites, the literature does not show any potential advantage to choosing surfactant-expressing strains from a particular environment, especially one that is contaminated (Bodour *et al.*, 2003, Satpute *et al.*, 2008, Thavasi *et al.*, 2011).

The advantage of using drop-collapse assay in the screening for surfactant-expression includes small volume requirement, ease of method and no specialised equipment requirement (Tugrul and Cansunar, 2005). Conversely, disadvantages include low sensitivity and in most cases, a requirement for further screening (Batista *et al.*, 2006, Bodour *et al.*, 2003, Płaza *et al.*, 2006, Youssef *et al.*, 2004). In this study, 58 LSTRA strains were further screened using quantitative tensiometry and of the 58 strains, 46 significantly reduced the surface tension of sterile KB* medium to a value of less than 30 mN/m (Figure **4.1**). However, the remaining 12 strains were statistically found to be an extension of the non-LSTRA strains thereby confirming the low precision power of the drop-collapse assay and in agreement with existing literature (Bodour and Maier, 2002, Lotfabad *et al.*, 2017, McInerney *et al.*, 1990, Morikawa *et al.*, 1993). Furthermore, a collection of 25 surfactant-expressing pseudomonads strains (Key strains) producing a limited range of very low surface tensions in KB* liquid cultures (24-26 mN/m) were selected for further study.

Although strains were isolated using PSA+CFC, the aim was to confirm these strains as pseudomonads using an Analytical Profiling Index (API kit) and ribosomal 16S DNA sequence (16S rDNA sequencing). The API 20e could identify up to 80% of the selected strains as pseudomonads, while the remaining 20% are unacceptable entries. Since API 20e is predominantly used to identify medically important bacterial strains, including members of the *Enterobactriaceae, Pseudomonas aeruginosa* and as such, it is not surprising to have up to 20% of the strains as unacceptable entries. This presumably means that the profile information of the

strains is not available on the data index (Kofli and Dayaon, 2010). Although up to 80% of the strains were identified as pseudomonads, other studies have shown that identification of environmental bacteria may be difficult using API 20e and that other techniques such as the DNA hybridisation and 16S rDNA sequencing are better when identifying environmental isolates (McLemore *et al.*, 2000). Thus, some strains were selected and were further characterised using the 16S rDNA sequence.

The 16S rDNA sequences of selected strains confirmed the key strains as pseudomonads with 99% homology (Appendix **A3.2**). It is interesting to note that some unacceptable data entries in the API kit were confirmed as pseudomonads using the 16S rDNA sequencing. Although the sequence BLAST resulted in identifying over 10 species with 99% identity, this could be connected to the extremely slow rate of evolution of 16S rDNA (Yamamoto *et al.*, 2000). Moreover, pseudomonads identification to species level cannot be achieved using the 16S rDNA sequencing alone (Yamamoto *et al.*, 1999). Other complementary methods such as DNA hybridisation or whole genome sequencing are useful when identifying pseudomonad strains to species level (Yamamoto and Harayama, 1998, Yamamoto *et al.*, 2000). However, analysing the 16S rDNA sequences using multiple sequence alignment analysis with the 16S gene sequence of 10 well-known pseudomonads in consideration of Chun *et al.*'s work (2007) resulted in high similarity with control sequences.

Similarly, further strain characterisation, using different growth-based and biochemical tests known for the genus pseudomonads (such as enzymes secretion including protease, lipase, gelatinase and resistance to antibiotics), confirmed the

strains as pseudomonads. These assays are particularly important in studying diversity among strains for future work or that, they can provide useful information when using these strains collection for some other purpose (e.g. isolation of an enzyme), or choosing which strains would be the easiest to grow. For the latter, the key strains showed diverse activity in protease secretion (90%), lipase secretion (87%), oxidase (93%), catalase (100%), gelatinase secretion (90%), and heavy metal resistance to mercury (7%). These assays are particularly important in differentiating strains when examining their potential in different biotechnological industries (Burger *et al.*, 2000, Robertson *et al.*, 2013). It is noted that some of these characteristics are novel and could be useful in different applications; for instance, a strain showing high activity towards protease secretion can be useful in agrochemical, leather and pharmaceutical industries, where they are used to hydrolyse proteins. Lipase enzyme comprises over 60% of the global enzyme market (Adrio and Demain, 2014, Erjavec *et al.*, 2012), while its secretion offers potentiality in fat breakdown and could therefore have uses in biodiesel generation, flavouring and agrochemical industries (Aravindan *et al.*, 2007, Hasan *et al.*, 2006). Other characteristics such as oxidase and catalase gelatinase activities are important in the food industries, while metal resistance indicates potentiality of applications in the treatment of heavy metal contamination and in the mining industries (Kirk *et al.*, 2002, Schrieber and Gareis, 2007, Uhlig, 1998).

Diversity amongst the key strains was investigated after Robertson *et al.*s' work (2013), with analysis of the phenotypic characterisation data being by use of a multivariate method of analysis and hierarchical cluster analysis approach. Using

these approaches, the key strains' phenotypic data were analysed and visualised as a constellation plot (Figure **3.4**). This allowed identification of all variations existing among the strains and established that the key strains were independent and did not contain biological replicates (Biological replicate means different strains measured across multiple assays). This signified the credibility of selection criteria that are affected by the contribution of factors such as sampling techniques and sample size (Lange *et al.*, 2015, Stackebrandt *et al.*, 1993). Future research will be to identify surfactant-expressing pseudomonads to species level using the whole genome approach or analysis of *gyr*B and *rpo*D sequences that are believe to close the resolution gap between 16S rDNA sequence analysis and DNA-DNA hybridization (Yamamoto *et al.*, 2000).

This research is interested in surfactant-expressing pseudomonads that produce a limited range of very low liquid surface tension reducing ability that may have potential application in biotechnology.

6.3 Pseudomonads surface tension and the prediction of limit

Surfactant characterisation using quantitative tensiometry has been reported in a number of studies (Balan *et al.*, 2017, Jemil *et al.*, 2016, Jha *et al.*, 2016, Shaligram *et al.*, 2016), and surveys of large collections of surfactant-expressing strains (Dong *et al.*, 2016, Fechtner *et al.*, 2011, Kryachko *et al.*, 2016, Mohammed *et al.*, 2015) reported surfactant activity ranging from 24-40 mN/m. Moreover, the use of probability distribution to study surfactant activity (Fechtner *et al.* (2011) and

Mohammed *et al.* (2014) has predicted a limit of 24 mN/m. This limit, also supported by Mohammed *et al.s'* analysis of 53 independent reported limits, appeared to be robust. However, this prediction was based on a less well-defined group of bacteria that where generally regarded as pseudomonads. Consequently, current studies using similar statistical approach confirm the limit among the pseudomonads group. This suggests that the limit is robust, even though Xia *et al.* (2011) report a limit for *P. aeruginosa* below the predicted value (22.9 mN/m). However, their report was limited to a single strain without statistical reproducibility.

Since KB* media contains growth components such as the proteose peptone which may influence surfactant activity, an attempt to establish if the aforementioned limit is influenced by growth and other chemical components present in the media was undertaken by analysing surface activity data generated from minimal M9Glu cultures. M9Glu is a culture medium that contains few additional salts that may not have effect on surfactant expression, but may interact with surfactants molecules to prevent their activity. Individual Distribution Identification (IDI) analysis resulted in the same limit of 24 mN/m (Figure **4.4**), thereby suggesting little or no influence of salts and other metabolites on the observed limit.

The positive correlation between surface tension data in KB* and that of M9Glu cultures further sustains the previous proposition that the limit is robust and is not attributable to growth media components. As a result, this work remains confident about the predicted limit of bacterial surfactants' strength. Although some strains show slight decrease in surface activity, it is obvious that factors such as pH, salt

concentration and temperature could significantly affect surfactant behaviour due to surfactants' amphipathic nature (Chen *et al.*, 2012, Khopade *et al.*, 2012, Vilela *et al.*, 2014, Xia *et al.*, 2011). Moreover, the possible interaction of surfactants with salt and pH was confirmed using semi-purified materials across pH and salt concentrations (Figure **5.9** and **5.10**). In fact, the decrease did not affect either surfactant expression or the minimum limit predicted.

The study presented in this thesis strongly suggests a robust limit that is not attributable to biochemical components present in the media. Moreover, the limit may have a biological explanation that is most probably the prevention of self-harm via non-polar interaction. This aspect remains a question for future research in understanding the bacterial limit, and why more active compounds are not produced. It is also suggested that further study related to this aspect could be to investigate if a similar limit could be achieved from another bacterial group (e.g. *Bacillus*).

6.4 Evidence for diversity in behaviour amongst strong surfactants

Initial reports of surfactant-expressing strains using phenotypic characterisation in Chapter **3** have shown that the key strains were diverse, even though they expressed limited range of surface activity. This could be supportive of a weakness in the hypothesis stating that they express similar surfactant. The best way to investigate surfactant diversity is to purify compounds and determine the chemical structure. An alternative approach could be whole genome sequencing for each

bacterial DNA to identify surfactants' genes, and then processing of the sequence using bioinformatics tools to infer surfactant structures (Deng *et al.*, 2016, Gogoi *et al.*, 2016, Janek *et al.*, 2010, Qiao and Shao, 2010, Shaligram *et al.*, 2016). However, this process is expensive, time consuming and impractical for bioprospecting studies.

The degree of chemical structural diversity amongst surfactants expressed by the pseudomonads has been reported by Van Hamme *et al.* (2006); Thanomsub *et al.* (2006) and reviewed by Raaijmakers *et al.* (2010). Although this group are phylogenetically similar, they are known to express several diverse classes of surfactants, including the cyclic lipo-peptides (CLPs) and rhamnolipids (D'aes *et al.*, 2010, Kuiper *et al.*, 2004). Studies characterising cyclic-lipopeptide chemical structure have shown the possibility of different structural analogues of surfactin with different sequences of amino acids and number of carbon atoms in the fatty acids (Bonmatin *et al.*, 2003, Hino *et al.*, 2001, Liu *et al.*, 2015). A study by Baumgart *et al.* (1991) reported three structural analogues of CLP that were different from each other and expressed by *B. subtilis* ATCC 21332 and OKB 105. Similarly, Kowall *et al.* (1998) reported 44 surfactin isoforms and their monomethyl and dimethyl esters expressed by *B. subtilis* OKB 105, thereby indicating strong structural diversity within surfactant subclasses.

Diversity among CLP varies depending upon structural composition, for instance the chemical structure of surfactins contains one residue of β-hydroxy fatty acid and seven residues of α-amino acids (Arima *et al.*, 1968, Hathout *et al.*, 2000). However, Iturins differs to surfactin by containing β-amino fatty acid as the lipidic part

(Bonmatin *et al.*, 2003, IWASE *et al.*, 2009). Similarly, surfactants differ in the number of carbon atoms forming the hydrophobic chain, which could typically range from 13 to 17 (Hosono and Suzuki, 1983, İkizler *et al.*, 2016, Isogai *et al.*, 1982, Vater *et al.*, 2002). Moreover, depending on the structure and size of the surfactant involved, surfactant behaviour varies. For instance, mono- and di-rhamnolipids and acidic and lactonic forms of sophorolipids differ in micelle sizes and thus are not identical in behaviour (Liu *et al.*, 2015, reviewed by Marchant and Banat, 2012). In addition, surfactins' molecular behaviour, including their morphology, changes with concentration, pH and metals ions (Han *et al.*, 2008). In addition, micropolarity and microviscosity were also observed to be affected, and these have a direct relationship to surfactant chemical structure (Li *et al.*, 2009, Osman *et al.*, 1998).

The direct link between surfactant chemical structure and behaviour could offer opportunities to study a large sample of surfactants using their oil-water behaviour and select them for further chemical structural analysis. This method has the potential of reducing the cost, while ensuring behavioural diverse group are selected for further testing. In this thesis, the chemical structural diversity of surfactants was investigated using a range of surfactant behavioural assays across pH and salt concentration. Individual ANOVA confirmed diversity among surfactants expressed by the key strains. Moreover, investigating diversity amongst surfactants using hierarchical cluster analysis showed a significant diversity among surfactants expressed by the key strains (Figure **5.7**). This confirms assumptions made earlier in this thesis and is in reflection of findings in existing literature on surfactant chemical

structural diversity (Baek *et al.*, 2007, Bouchez Naïtali *et al.*, 1999, Gudiña *et al.*, 2013, Kruijt *et al.*, 2009, Marqués *et al.*, 2009). Further investigation of diversity among surfactants close to one another in the constellation plot (Figure **5.7**), using semi-purified material across pH and salt concentration, resulted in the revelation of minimal differences existing between them. Moreover, failure to obtain a semi-purified material from strains 5 and 20 indicated that the strains were expressing an entirely different class of surfactant, and thus confirming chemical structural diversity among the key strains. This finding conforms to findings by Ishigami *et al.* (1987) and Champion *et al.* (1995), who argued that the lamellar morphology of rhamnolipid is dependent on the pH and that it could change to vesicular and finally micellar as pH is increased. This was confirmed by Shin *et al.* (2008) and reflects results that show the effect of salinity or ionic strength on surfactants on solubility (Ochoa-Loza *et al.*, 2001, Rosen and Kunjappu, 2012, Wang *et al.*, 2007).

The diversity constellation plot has grouped surfactants expressed by key strains into 5 major groups that signify 5 structurally diverse surfactants (Figure **5.7**). Although the variation observed between groups may be high and surfactants that belong to the same group may differ, possible minimal variation could be the result of slight changes in amino acids or the length of carbon. This could result in substantial consequences for the overall behaviour of the surfactant due to changes in the hydrophilic-lipophilic balance (Perfumo *et al.*, 2010). This assumption was confirmed using semi-purified surfactants, where it was thought growth media components contributed to the slight changes experienced in surfactant activity between closely related strains. Furthermore, the results reveal slight differences

observed between surfactants belonging to the same group (Figure **5.9** and **5.10**), thereby confirming the slight diversity seen in surfactant chemical structure as reported by Abdel-Mawgoud *et al.* (2010); Bonmatin *et al.* (2003); Ongena and Jacques (2008) and Roongsawang *et al.* (2010).

The significance of these findings will be utilised as a potential screening tool for selecting novel surfactants for further analysis. For instance, this method could guide researchers selecting surfactants with different chemical structural behaviour for further analysis and testing in biotechnology, especially in a study with a modest budget and time constrains. In such a study, selection of one strain from each of the 5 clusters for further analysis could perhaps result in 5 structurally diverse surfactants that could be tested in biotechnology.

There is interest in identifying novel strains for use in different biotechnological applications. Previous studies have identified surfactant roles in agriculture, food, pharmaceutical and oil remediation (Marchant and Banat, 2012, reviewed by Raaijmakers *et al.*, 2010, Tran *et al.*, 2007). Studies conducted in this aspect predominantly investigate surfactant potential, including their growth on different carbon substrate by single or multiple strains (Gong *et al.*, 2015, Reddy *et al.*, 2016, Wu *et al.*, 2008), even though substrate types and surfactant purity degrees are found to influence surfactant activity (Reddy *et al.*, 2016).

In this thesis, a different approach was used to study the behaviour of surfactants in 4 representative oils (diesel and used-lubricating oil from petroleum industries, vegetable from food and mineral from biotechnological industries), with the aim of

finding surfactants with a functional role in food, remediation and other biotechnological applications**.** The HCA constellation plotting behaviour in different oil types and different conditions resulted in more diversity among the oil types than the conditions (buffer) used (Figure **5.8**). This finding indicates that surfactants have different behaviours in oil, and suggests some surfactants may be better-utilised in specific applications. For instance, some surfactants have higher activity in vegetable oil than in used-lubricating oil, and this could perhaps mean potential applications within the food industries. Furthermore, it conforms to with the existing literature that report on the potential application of surfactants in food, remediation and other biotechnological areas (Gudiña *et al.*, 2013, Sachdev and Cameotra, 2013, Salim *et al.*, 2014).

The HCA of diversity and behaviour were strongly supported by the generalised linear modelling techniques (GLM). GLM has been a useful tool for measuring relationships and prediction of effect sources (Guisan and Theurillat, 2000). Modelling the oil-water behaviour of surfactants resulted in a low goodness of fit value (R^2), thereby indicating a significant relationship between the data sets. Similarly, the effect test resulted in identifying the variation observed in the oil, conditions and replicates. For the latter, this could be due to difficulty in obtaining a uniform diameter in oil displacement. These results support data in this thesis by confirming its credibility and the manner in which the assays where conducted (Austin, 2002). Further studies in this regard could be to select a representative of each group and characterise it using chemical structure or molecular base techniques, and to then assess their performance in different applications.

Surfactant chemical structural characterisation could be achieved using the techniques described in Section **1.2.** The molecular base technique is feasible using homology-based techniques involving identification of genes responsible for surfactants expression, or by whole genome sequencing (Giordano *et al.*, 2002).

A preliminary study was conducted to investigate the mechanisms of surfactant-expression by the best performing strain (strain 1) using techniques noted in Stephen *et al.* (2007). Transposon mutagenesis resulted in the identification of nine transposon mutants after screening 5025 isolates from the mini-Tn library. Although initial attempts to obtain affected gene sequence information failed, the mutants and the wild type were further characterised using assays to assess their phenotypes (see Appendix **A6.1** for Materials and Methods).

De Bruijn and Raaijmakers's study (2008) shows a direct relationship between loss of surfactant production and swarming motility by *Pseudomonas* spp.. In this study, phenotypic characterisation of the nine mutants plus the wild type was ascertained with motility assays (swarming, swimming and twitching) and quantitative surface activity. Initial observation based upon their appearance and swimming showed that the mutants differed with one another in their fluorescent appearance and in the swimming assays (see Appendix **A6.2**). All the mutants lost their ability to swarm but remained positive to twitching character (Appendix **A6.3** and **A6.4**). This suggests multiple genes are involved in surfactant expression (de Bruijn *et al.*, 2007, de Bruijn and Raaijmakers, 2009b, De Souza *et al.*, 2003). Moreover, quantitative surface activity measurement of liquid culture supernatant of the mutants and the wild type indicates that only the wild type significantly reduced the surface tension

of sterile KB* culture, while the mutants did not (see Appendix **A6.5**). This further confirms the loss of regulatory function of the genes responsible for surfactant expression. Further study is needed to identify the genes responsible for surfactant expression by identifying the transposon insert using homology-based techniques.

Moreover, the best performing strain (strain 1) was sent to MicrobesNG, UK for whole genome sequencing. This may be useful in gene identification process as well in some homology-based bioinformatics analysis.

6.5 Thesis conclusion

The work undertaken in this thesis contains significant advances in our understanding of the behaviour of surfactant-expressed by the pseudomonads. This work shows that surfactant expression and the limit of activity observed are not influenced by salts or other chemicals present in the growth media. Moreover, the work demonstrates significant chemical structural variation within a collection of high-performing surfactants expressed by *Pseudomonas* spp. strains producing a narrow range of surface tensions in liquid cultures. This finding indicates that bio-prospecting for new bacterial surfactants by screening for only the most surface-active compounds is likely to reveal a range of different surfactants having varied behaviours in air-water and water-oil mixtures.

Finally, the constellation dendrogram produced using data from simple behavioural assays, such as those used in this thesis, provides a useful tool for identifying surfactants for future testing.

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Appendices

Appendix A3

Additional information for Chapter 3

Appendix A3.1: Phenotypic assay results for the 30 key strains

Initial strain No.	Final strain No.	Sample point	Oxidase	Catalase	Gelatinase activity	Lipase secretion	Swarming	NaCl	Milk	Swimming	Twitching	MH Agar	Mucoid	Flourescent	Mercury	KB* acidity	Glucose	Tetracycline resistance	Kenamycine resistance	M9Suc	Strep S (disc)	Tetr S (disc)	Chlo S	Nalidixic acid	Col S	Ts S	Streptomycin S	Temperature 42 °C	Temperature 28 °C
Strain 40	**1**	AA	p	p	p	P	p	p	p	p	p	n	p	n	n	p	p	n	n	p	p	p	n	n	p	n	n	n	n
Strain 34	**2**	T	p	p	p	P	p	p	p	p	n	n	n	p	n	p	p	n	n	n	n	p	n	n	p	n	n	n	n
Strain 7	**3**	D	p	p	p	P	p	p	p	p	p	n	n	p	n	p	p	n	n	n	p	p	n	p	n	n	n	n	p
Strain 42	**4**	AB	p	p	p	P	p	p	p	p	p	n	p	n	n	p	p	n	n	p	p	n	n	n	p	n	p	n	n
Strain 13	**5**	H	p	p	p	P	p	p	p	p	p	n	n	p	n	p	p	n	n	n	p	p	n	p	n	n	p	n	n
Strain 15	**6**	H	p	p	p	P	p	p	p	p	n	n	n	p	n	p	p	n	n	n	p	p	n	p	n	n	p	n	n
Strain 33	**7**	T	p	p	p	P	p	p	p	n	n	n	n	p	n	p	p	n	n	n	p	p	n	n	n	n	p	n	n
Strain 36	**8**	T	p	p	p	p	p	p	p	p	n	n	n	p	n	p	p	n	n	n	p	p	n	p	n	n	n	n	p

Strain 3	**9**	B	p	p	p	P	p	p	p	n	p	n	n	n	p	p	p	n	n	p	p	p	n	p	p	n	n	n	n
Strain 31	**10**	T	p	p	p	P	p	p	p	p	n	n	n	p	n	p	p	n	n	n	p	p	n	n	n	n	p	n	n
Strain 37	**11**	T	p	p	p	P	p	p	p	p	n	n	n	p	n	p	p	n	n	n	p	n	n	p	n	n	p	n	n
Strain 14	**12**	H	p	p	p	P	p	p	p	p	n	n	n	n	n	p	p	n	n	n	p	p	n	p	n	n	p	n	n
Strain 12	**13**	G	p	p	p	P	p	p	p	p	n	n	n	n	n	p	p	n	n	n	p	p	n	p	n	n	n	n	n
Strain 23	**14**	L	n	p	p	P	n	p	p	n	p	n	n	p	p	n	p	n	p	n	p	p	p	p	p	n	n	n	p
Strain 27	**15**	O	p	p	p	P	p	p	p	p	n	n	n	p	p	p	p	n	n	n	p	p	n	p	n	n	p	n	n
Strain 20	**16**	J	p	p	p	N	p	p	p	p	p	n	p	p	p	p	p	n	n	n	p	p	n	n	p	n	p	p	p
Strain 43	**17**	AF	p	p	p	P	n	p	p	p	p	n	p	n	p	p	p	n	n	p	n	p	p	n	p	n	n	n	n
Strain 30	**18**	O	p	p	p	P	p	p	p	p	n	n	p	p	n	p	p	n	n	n	p	p	n	p	n	n	n	p	p
Strain 41	**19**	AA	p	p	p	P	n	p	p	p	p	n	p	p	n	p	p	n	n	p	p	p	n	p	n	n	n	n	n
Strain 52	**20**	AK	p	p	p	P	p	p	p	p	n	n	p	p	n	p	p	n	n	p	p	p	n	p	p	n	n	n	n
Strain 45	**21**	AH	p	p	p	p	p	p	p	p	p	n	p	p	p	p	p	n	n	n	p	p	n	p	n	n	n	n	n
Strain	**22**	O	n	p	p	N	p	p	p	p	p	n	p	p	p	n	p	n	p	p	p	p	p	n	p	n	n	n	n

29																												
Strain 9	**23**	G	p	p	p	N	p	p	p	p	p	n	p	p	p	p	p	n	n	n	p	p	n	p	p	n	p	n	n
Strain 32	**24**	T	p	p	p	P	n	p	p	p	p	n	n	p	n	p	p	n	n	n	p	p	n	p	n	n	p	n	n
Strain 51	**25**	AJ	p	p	p	P	p	p	p	p	n	n	n	n	n	p	p	n	n	n	p	p	n	p	n	n	p	n	n
Control 1	**c1**	Y	p	p	p	N	n	p	p	p	p	n	p	n	p	p	p	n	n	p	n	p	n	n	p	n	n	n	n
Control 2	**c2**	Y	p	p	n	N	n	n	n	p	p	n	n	n	p	p	p	n	n	n	p	p	p	p	n	n	p	n	n
Control 3	**c3**	Z	p	p	p	N	p	p	n	p	p	n	n	p	p	p	p	n	n	p	n	p	p	p	n	p	p	n	n
Control 4	**c4**	AB	p	p	n	N	n	n	n	p	p	n	n	n	p	p	p	n	n	n	p	p	n	p	p	n	n	n	n
Control 5	**c5**	AA	p	p	n	N	n	n	n	n	p	n	n	n	n	p	p	n	n	n	p	p	n	p	p	n	p	n	p

Key: MH Agar = Muller-Hinton; TetR = Tetracycline resistance; KenR = Kanamycin resistance; M9S = Minimal medium with sucrose; Strep S (disc) = streptomycin; Chlo S = chloramphenicol; NalI S = nalidixic acid; Col S = colistrin sulphate; Ts S = cotrimoxazole; St S = sulphatriad; T 42 = temperature 42^{o}C; TR 28 = temperature 28^{o}C.

Appendix A3.2: 16S rDNA sequences for selected strains

Strain 1

5’GAATTCGCCCTTTTAGCTCCACCTCGCGGCTTGGCAACCCTTTGTACCGACCATTGCAGCA
CGTGTGTAGCCCAGGCCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCGGTT
TGTCACCGGCAGTCTCCTTAGAGTGCCCACCATAACGTGCTGGTAACTAAGGACAAGGGTT
GCGCTCGTTACGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCA
CCTGTCTCAATGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCATTGGATGTCAAGGCC
TGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCC
GTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCAACTTAATGCGTTAG
CTGCGCCACTAAGAGCTCAAGGCTCCCAACGGCTAGTTGACATCGTTTACGGCGTGGACTAC
CAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGT
GGTCGCCTTCGCCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTC
CACCACCCTCTACCATACTCTAGCTCGACAGTTTTGAATGCAGTTCCCAGGTTGAGCCCGGG
GATTTCACATCCAACTTAACGAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACG
CTTGCACCCTCTGTATTACCGCGGCTGCTGGCACAGAGTTAGCCGGTGCTTATTCTGTCGGT
AACGTCAAAACAGCAACGTATTAAGTTACTGCCCTTCCTCCCAACTTAAAGTGCTTTACAATC
CGAAAGACCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAA
CCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGT
GCTGCAATGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAAAAGGGCGAATTC
3’

Strain 2

5’GAATTCGCCCTTTTTAGCTTCCACCTCGCGGCTTGGCAACCCTTCTGTACCGACCCATTGTA
GCCACGTGTGTAGCCCCAGGCCGTAAGGGCCCATGATGACTTGACGTCATCCCCACCTTCCT
CCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCACCATAACGTGCTGGTAACTAAGGACAA
GGGTTGCGCTCGTTACGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATG
CAGCACCTGTCTCAATGTTCCCGAAGGCACCAATCTATCTCTAGAAAGTTCATTGGATGTCAA
GGCCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGC
CCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCAACTTAATGCG
TTAGCTGCGCCACTAAAAGCTCAAGGCTTCCAACGGCTAGTTGACATCGTTTACGGCGTGGA
CTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATTAGTCC
AGGTGGTCGCCTTCGCCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAA
ATTCCACCACCCTCTACCATACTCTAGTCAGTCAGTTTTGAATGCAGTTCCCAGGTTGAGCCC
GGGGATTTCACATCCAACTTAACAAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTA
ACGCTTGCACCCTCTGTATTACCGCGGCTGCTGGCACAGAGTTAGCCGGTGCTTATTCTGTC
GGTAACGTCAAAACAATCACGTATTAGGTAACTGCCCTTCCTCCCAACTTAAAGTGCTTTACA
ATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAATA
TTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGACTGATCATC
CTCTCAGACCAAGGGCGAATTC3’

Strain 3

5’GAATTCGCCCTTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGAC
TCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATG
CCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCATTA
ACCTAATACGTTAGTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGC
AGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGT
AGGTGGTTTGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACT
GACTGACTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGAT
ATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTAATACTGACACTGAGGTGCG
AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAAC
TAGCCGTTGGAAGCCTTGAGCTTTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGG
GAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGGGGTGGAG
CATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAATGAACTTT
CTAGAGATAGATTGGTGCCTTCGGGAACATTGAGACAGGTGCTGCATGGCTGTCGTCAGTT
CGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGC
ACGTAATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGA
CGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGA
GGGTTGCCAAGCCGCGAGGTGGAGCTAAAAGGGCGAATTC3’

Strain 4

5’GAATTCGCCCTTTTAGCTCCACCTCGCGGCTTGGCAACCCTTTGTACCGACCATTGTAGCAC
GTGTGTAGCCCAGGCCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTT
GTCACCGGCAGTCTCCTTAGAGTGCCCACCATAACGTGCTGGTAACTAAGGACAAGGGTTG
CGCTCGTTACGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACC
TGTCTCAATGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCATTGGATGTCAAGGCCTG
GTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGT
CAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCAACTTAATGCGTTAGCT
GCGCCACTAAGAGCTCAAGGCTCCCAACGGCTAGTTGACATCGTTTACGGCGTGGACTACC
AGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTG
GTCGCCTTCGCCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAGATTCC
ACCACCCTCTACCATACTCTAGCTCGACAGTTTTGAATGCAGTTCCCAGGTTGAGCCCGGGG
ATTTCACATCCAACTTAACGAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACGC
TTGCACCCTCTGTATTACCGCGGCTGCTGGCACAGAGTTAGCCGGTGCTTATTCTGTCGGTA
ACGTCAAAACAGCAACGTATTAAGTTACTGCCCTTCCTCCCAACTTAAAGTGCTTTACAATCC
GAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCCC
CACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGACTGATCATCCTCTC
AGACCAAGGGCGAATTC3’

Strain 5

5’GAATTCGCCCTTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGAC
TCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATG
CCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCATTA
ACCTAATACGTTAGTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGC
AGCCGCGGTAATACAGAGGGTGCAAGTGTTAATCGGAATTACTGGGCGTAAAGCGCGCGT
AGGTGGTTTGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACT
GACTGACTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGAT
ATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTAATACTGACACTGAGGTGCG
AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAAC
TAGCCGTTGGAAGCCTTGAGCTTTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGG
GAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAG
CATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAATGAACTTT
CTAGAGATAGATTGGTGCCTTCGGGAACATTGAGACAGGTGCTGCATGGCTGTCGTCAGCT
CGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGC
ACGTTATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGA
CGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGA
GGGTTGCCAAGCCGCGAGGTGGAGCTAAAAGGGCGAATTC3’

Strain 15

5’GAATTCGCCCTTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGAC
TCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATG
CCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCATTA
ACCTAATACGTTAGTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGC
AGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGT
AGGTGGTTTGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACT
GACTGACTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGAT
ATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTAATACTGACACTGAGGTGCG
AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAAC
TAGCCGTTGGAAGCCTTGAGCTTTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGG
GAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAG
CATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAATGAACTTT
CTAGAGATAGATTGGTGCCTTCGGGAACATTGAGACAGGTGCTGCATGGCTGTCGTCAGCT
CGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGC
ACGTAATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGA
CGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGA
GGGTTGCCAAGCCGCGAGGTGGAGCTAAAGGGCGAATTC3’

Strain 20

5’GAATTCGCCCTTGGTCTGAGAGGATGATCAGTCACACCGGAACTGAGACACGGTCCAGAC
TCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCCGATCCAGCCATG
CCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTT
ACCTAATACGTGATTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGC
AGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGT
AGGTGGTTTGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACT
GACTGACTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGAT
ATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCG
AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAAC
TAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGG
GAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAG
CATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAATGAACTTT
CTAGAGATAGATTGGTGCCTTCGGGAACATTGAGACAGGTGCTGCATGGCTGTCGTCAGCT
CGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGC
ACGTTATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGA
CGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGA
GGGTTGCCAAGCCGCGAGGTGGAGCTAAAAGGGCGAATTC3’

Strain 25

5’GAATTCGCCCTTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGAC
TCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATG
CCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCATTA
ACCTAATACGTTAGTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGC
AGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAGGCGCGCGT
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GACTGACTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGAT
ATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTAATACTGACACTGAGGTGCG
AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAAC
TAGCCGTTGGAAGCCTTGAGCTTTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGG
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CATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAATGAACTTT
CTAGAGATAGATTGGTGCCTTCGGGAACATTGAGACAGGTGCTGCATGGCTGTCGTCAGCT
CGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGC
ATGTCATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGA
CGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGA
GGGTTGCCAAGCCGCGAGGTGGAGCTAAAAGGGCGAATTC3’

Strain C1

5’GAATTCGCCCTTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGAC
TCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATG
CCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCATTA
ACCTAATACGTTGGTGTCTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCGGC
AGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGT
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GACAAGCTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGA
TATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGC
GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCA
ACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTG
GGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGG
AGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAATGAATC
TGCTAGAGATAGCAGAGTGCCTTCGGGAACATTGAGACAGGTGCTGCATGGCTGTCGTCAG
CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCA
GCACGTAATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGAT
GACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACA
GAGGGTTGCCAAGCCGCGAGGTGGAGCTAAAAGGGCGAATTC3’

Appendix A3.3: Ten closest homolog of the 16s rDNA sequence for the selected strains

Strain	Closest *Pseudomonas* spp. homology	Identity (%)
1	*Pseudomonas baetica* a390	99
	Pseudomonas brassicacearum CIP 109457	99
	Pseudomonas frederiksbergensis DSM 13022	99
	Pseudomonas frederiksbergensis JAJ28	99
	Pseudomonas helmanticensis OHA11	99
	Pseudomonas lini DLE411J	99
	Pseudomonas migulae NBRC 103157	99
	Pseudomonas migulae CIP 105470	99
	Pseudomonas umsongensis Ps 3-10	99
	Pseudomonas thivervalensis SBK26	99

Strain	Closest *Pseudomonas* spp. homology	Identity (%)
2	*Pseudomonas antarctica* CMS 35	99
	Pseudomonas extremaustralis 14-3	99
	Pseudomonas lurida P 513/18	99
	Pseudomonas meridiana CMS 38	99
	Pseudomonas marginalis ICMP 3553	99
	Pseudomonas poae P 527/13	99
	Pseudomonas simiae OLi	99
	Pseudomonas tolaasii ATCC 33618	99
	Pseudomonas tolaasii NBRC 103163	99
	Pseudomonas trivialis P 513/19	99

Strain	Closest *Pseudomonas* spp. homology	Identity (%)
3	*Pseudomonas fluorescens* NBRC 14160	99
	Pseudomonas fluorescens CCM 2115	99
	Pseudomonas fluorescens ATCC 13525	99
	Pseudomonas fluorescens Rhodes 28/5	99
	Pseudomonas lurida P 513/18	99
	Pseudomonas poae RE*1-1-14	99
	Pseudomonas poae P 527/13	99
	Pseudomonas salomonii CFBP 2022	99
	Pseudomonas simiae Oli	99
	Pseudomonas trivialis P 513/19	99

Strain	Closest *Pseudomonas* spp. homology	Identity (%)
4	*Pseudomonas baetica* a390	99
	Pseudomonas brassicacearum CIP 109457	99
	Pseudomonas brassicacearum NFM421	99
	Pseudomonas frederiksbergensis DSM 13022	99
	Pseudomonas frederiksbergensis JAJ28	99
	Pseudomonas helmanticensis OHA11	99
	Pseudomonas lini DLE411J	99
	Pseudomonas migulae NBRC 103157	99
	Pseudomonas migulae CIP 105470	99
	Pseudomonas umsongensis Ps 3-10	99

Strain	Closest *Pseudomonas* spp. homology	Identity (%)
5	*Pseudomonas fluorescens* NBRC 14160	99
	Pseudomonas fluorescens CCM 2115	99
	Pseudomonas fluorescens ATCC 13525	99
	Pseudomonas fluorescens M. Rhodes 28/5	99
	Pseudomonas lurida P 513/18	99
	*Pseudomonas poae RE*1-1-14*	99
	Pseudomonas poae P 527/13	99
	Pseudomonas trivialis P 513/19	99
	Pseudomonas salomonii CFBP 2022	99
	Pseudomonas simiae OLi	99

Strain	Closest *Pseudomonas* spp. homology	Identity (%)
15	*Pseudomonas fluorescens* ATCC 13525	99
	Pseudomonas fluorescens NBRC 14160	99
	Pseudomonas fluorescens CCM 2115	99
	Pseudomonas fluorescens M. Rhodes 28/5	99
	Pseudomonas lurida P 513/18	99
	Pseudomonas poae P 527/13	99
	Pseudomonas poae RE*1-1-14	99
	Pseudomonas simiae OLi	99
	Pseudomonas salomonii CFBP 2022	99
	Pseudomonas trivialis P 513/19	99

Strain	Closest *Pseudomonas* spp. homology	Identity (%)
20	*Pseudomonas antarctica* CMS 35	99
	Pseudomonas extremaustralis 14-3	99
	Pseudomonas marginalis ICMP 3553	99
	Pseudomonas meridiana CMS 38	99
	Pseudomonas poae P 527/13	99
	*Pseudomonas poae RE*1-1-14*	99
	Pseudomonas rhodesiae CIP 104664	99
	Pseudomonas simiae OLi	99
	Pseudomonas trivialis P 513/19	99
	Pseudomonas veronii CIP 104663	99

Strain	Closest *Pseudomonas* spp. homology	Identity (%)
25	*Pseudomonas fluorescens* CCM 2115	99
	Pseudomonas fluorescens NBRC 14160	99
	Pseudomonas fluorescens ATCC 13525	99
	Pseudomonas fluorescens M. Rhodes 28/5	99
	Pseudomonas lurida P 513/18	99
	Pseudomonas poae RE*1-1-14	99
	Pseudomonas poae P 527/13	99
	Pseudomonas simiae OLi	99
	Pseudomonas salomonii CFBP 2022	99
	Pseudomonas trivialis P 513/19	99

Strain	Closest *Pseudomonas* spp. homology	Identity (%)
C1	*Pseudomonas arsenicoxydans* VC-1	99
	Pseudomonas corrugata	99
	Pseudomonas corrugata Slade 939/1	99
	Pseudomonas fluorescens NBRC 14160	99
	Pseudomonas fluorescens CCM 2115	99
	Pseudomonas fluorescens ATCC 13525	99
	Pseudomonas fluorescens M. Rhodes 28/5	99
	Pseudomonas kilonensis 520-20	99
	Pseudomonas mandelii NBRC 103147	99
	Pseudomonas thivervalensis SBK26	99

Appendix A4

Additional information for Chapter 4

Appendix 4.1: Mean liquid surface tension for the 58 drop-collapse positive strains plus 20 negative control

New No.	Old No.	Mean ± SE	New No.	Old No.	Mean ± SE
1	Strain 40	25.04 ± 0.09	36	Strain 19	26.48 ± 0.10
2	Strain 34	25.20 ± 0.12	37	Strain 22	26.54 ± 0.16
3	Strain 7	25.21 ± 0.08	38	Strain 55	26.83 ± 0.04
4	Strain 42	25.23 ± 0.08	39	Strain 21	27.02 ± 0.56
5	Strain 13	25.24 ± 0.01	40	Strain 25	27.39 ± 0.22
6	Strain 15	25.26 ± 0.03	41	Strain 18	27.54 ± 0.46
7	Strain 33	25.26 ± 0.16	42	Strain 17	27.80 ± 0.49
8	Strain 36	25.26 ± 0.12	43	Strain 39	28.52 ± 0 .08
9	Strain 3	25.29 ± 0.11	44	Strain 10	28.79 ± 0.05
10	Strain 31	25.30 ± 0.08	45	Strain 49	30.25 ± 0.20
11	Strain 37	25.31 ± 0.09	46	Strain 1	32.30 ± 1.49
12	Strain 14	25.32 ± 0.04	47	Strain 56	35.09 ± 0.82
13	Strain 12	25.39 ± 0.03	48	Strain 28	42.88 ± 1.14
14	Strain 23	25.43 ± 0.07	49	Strain 16	46.21 ± 3.55
15	Strain 27	25.46 ± 0.07	50	Strain 5	49.24 ± 0.40
16	Strain 20	25.51 ± 0.05	51	Strain 4	49.70 ± 0.27
17	Strain 43	25.57 ± 0.07	52	Strain 2	49.74 ± 0.64
18	Strain 30	25.57 ± 0.07	53	Strain 11	50.13 ± 1.60
19	Strain 41	25.59 ± 0.08	54	Strain 57	50.28 ± 0.97
20	Strain 52	25.60 ± 0.06	55	Strain 6	52.12 ± 0.23
21	Strain 45	25.62 ± 0.09	56	Strain 50	54.75 ± 0.28
22	Strain 29	25.71 ± 0.06	57	Strain 46	56.14 ± 1.22
23	Strain 9	25.74 ± 0.21	58	Strain 47	56.94 ± 0.54
24	Strain 32	25.77 ± 0.09	C1	Control 1	58.77 ± 0.32
25	Strain 51	25.83 ± 0.04	C2	Control 2	58.49 ± 0.12
26	Strain 48	25.97 ± 0.08	C3	Control 3	52.95 ± 0.91
27	Strain 58	26.00 ± 0.08	C4	Control 4	58.58 ± 0.45
28	Strain 54	26.03 ± 0.04	C5	Control 5	57.13 ± 1.17
29	Strain 53	26.04 ± 0.10	C6	Control 6	57.52 ± 0.17
30	Strain 44	26.06 ± 0.56	C7	Control 7	61.36 ± 0.12
31	Strain 24	26.35 ± 0.08	C8	Control 8	50.49 ± 0.64
32	Strain 26	26.36 ± 0.11	C9	Control 9	49.09 ± 0.54
33	Strain 38	26.41 ± 0.09	C10	Control 10	59.61 ± 0.20
34	Strain 8	26.42 ± 0.22			
35	Strain 35	26.44 ± 0.07			

New No.	Old No.	Mean ± SE
C11	Control 11	59.50 ± 0.15
C12	Control 12	59.06 ± 0.35
C13	Control 13	59.09 ± 0.21
C14	Control 14	53.49 ± 0.26
C15	Control 15	54.23 ± 0.30
C16	Control 16	49.08 ± 0.28
C17	Control 17	29.27 ± 0.09
C18	Control 18	59.95 ± 0.33
C19	Control 19	59.11 ± 0.08
C20	Control 20	39.95 ± 4.65
KB*	KB*	53.97 ± 0.31

Appendix A4.2: post hoc multiple comparison tests and the resulting homogenous group

Old strain No.	N	Subset 1	2	3	4	5	6	7	8	9	10
Strain 40	4	25.03600									
Strain 34	4	25.19925									
Strain 7	4	25.20650									
Strain 42	4	25.22600									
Strain 13	4	25.23450									
Strain 15	4	25.25475									
Strain 33	4	25.25925									
Strain 36	4	25.26100									
Strain 3	4	25.29100									
Strain 31	4	25.30400									
Strain 37	4	25.30850									
Strain 14	4	25.31800									
Strain 12	4	25.39025									
Strain 23	4	25.43200									
Strain 27	4	25.45450									
Strain 20	4	25.50675									
Strain 43	4	25.56775									
Strain 30	4	25.57100									
Strain 41	4	25.59275									
Strain 52	4	25.60200									
Strain 45	4	25.61625									
Strain 58	4	25.69900									
Strain 29	4	25.70500									
Strain 9	4	25.73625									
Strain 32	4	25.76900									
Strain 51	4	25.83225									
Strain 48	4	25.96975									
Strain 54	4	26.02950									
Strain 53	4	26.03475									
Strain 44	4	26.05450									
Strain 24	4	26.35150	26.35150								
Strain 26	4	26.35525	26.35525								
Strain 38	4	26.40675	26.40675								
Strain 8	4	26.41575	26.41575								
Strain 35	4	26.43875	26.43875								
Strain 19	4	26.48000	26.48000								
Strain 22	4	26.54025	26.54025								
Strain 55	4	26.82725	26.82725								
Strain 21	4	27.01525	27.01525								
Strain 25	4	27.39225	27.39225								
Strain 18	4	27.54350	27.54350								
Strain 17	4	27.79950	27.79950								
Strain 39	4	28.52150	28.52150	28.52150							
Strain 10	4	28.79150	28.79150	28.79150							
Strain 49	4		30.24775	30.24775							
Strain 1	4			32.30100	32.30100						
Strain 56	4				35.08600						
Strain 28	4					42.88125					
Strain 16	4					46.21375	46.21375				
Strain 5	4						49.24225	49.24225			
Strain 4	4						49.70350	49.70350	49.70350		
Strain 2	4						49.73550	49.73550	49.73550		
Strain 11	4						50.12525	50.12525	50.12525		
Strain 57	4							50.27450	50.27450		
Strain 6	4							52.10925	52.10925	52.10925	
ST KB*	20								53.55230	53.55230	53.55230
Strain 50	4									54.74950	54.74950
Strain 46	4										56.71850
Strain 47	4										56.94250
Sig.		.103	.065	.096	.784	.326	.062	.721	.076	.877	.285

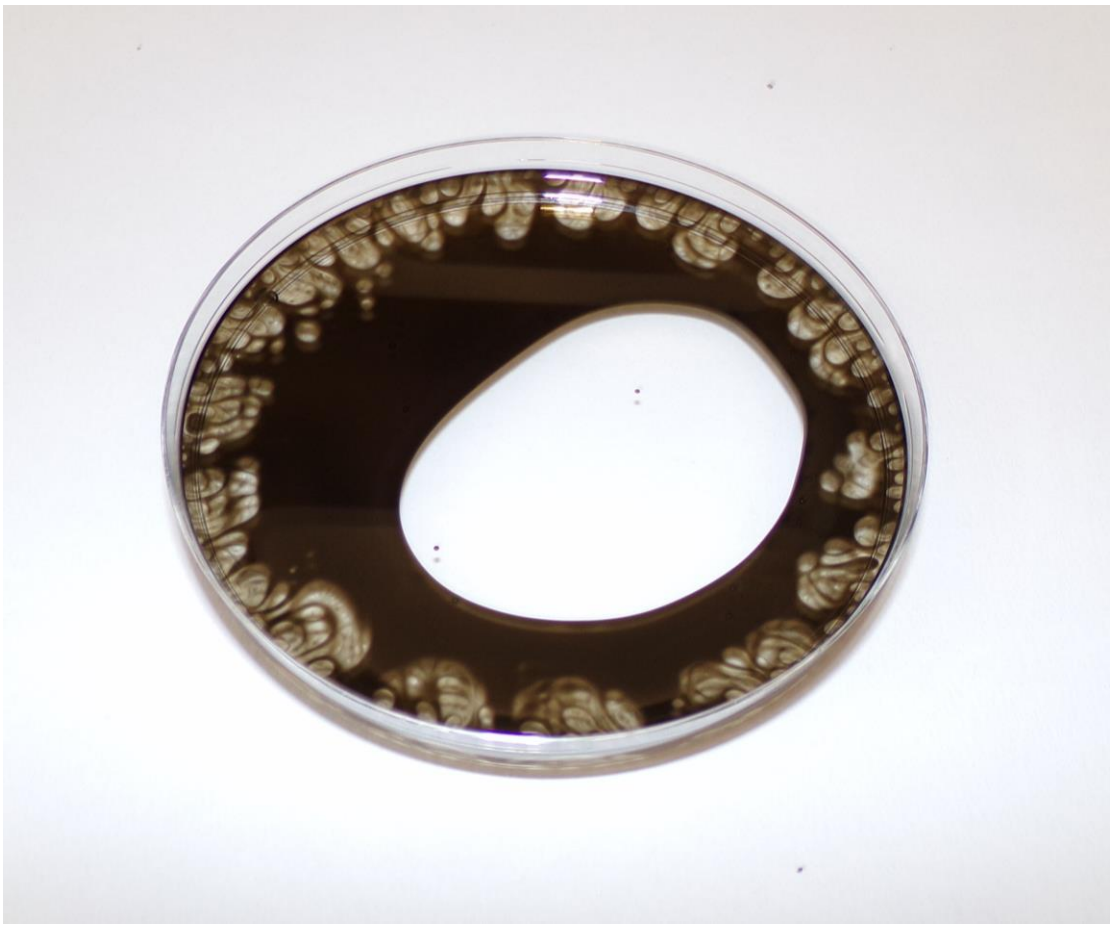
Appendix A5

Additional information for Chapter 5

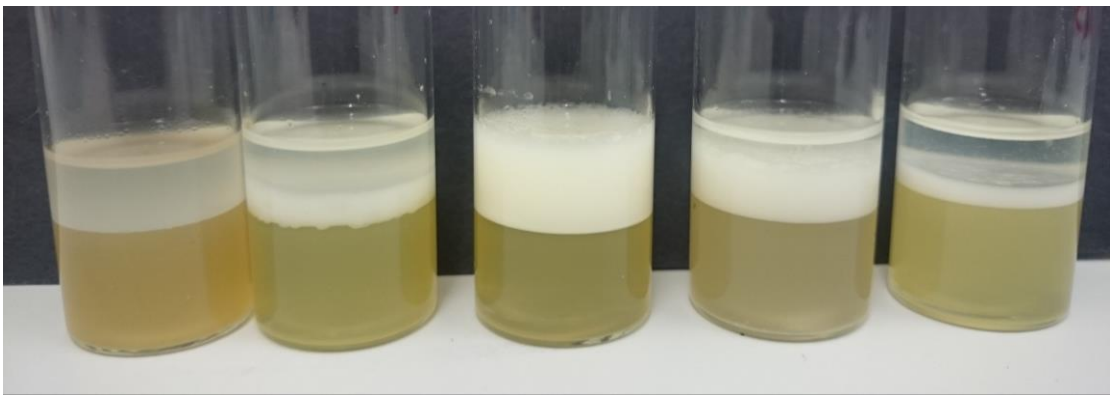
Appendix A5.1: ANOVA p-values for the 12 independent oil displacement assays

Condition	Strain Df	Test value	Significant value
200mM salt overlaid with diesel oil	29	85.001	<0.0001
pH 8 overlaid with diesel oil	29	98.845	<0.0001
pH 6 overlaid with diesel oil	29	51.116	<0.0001
200mM salt overlaid with mineral oil	29	54.367	<0.0001
pH 8 overlaid with mineral oil	29	35.571	<0.0001
pH 6 overlaid with mineral oil	29	20.116	<0.0001
200mM salt overlaid with ULO	29	17.946	<0.0001
pH 8 overlaid with ULO	29	20.770	<0.0001
pH 6 overlaid with ULO	29	12.044	<0.0001
200mM salt overlaid with vegetable oil	29	23.287	<0.0001
pH 8 overlaid with vegetable oil	29	25.520	<0.0001
pH 6 overlaid with vegetable oil	29	14.896	<0.0001

Key: Df = degree of freedom; ULO = used lubricating oil.
200 mM salt, pH 8 and 6 provided the aqueous phase while the four oils (diesel, mineral, ULO and vegetable) provided the thin oil layer for the assays.



Appendix A5.2: Surfactant behaviours depend on the chemical structure of the compound. Shown here is the impact of a small drop of surfactant-containing culture on a thin film of used lubricating oil.



Appendix A5.3: Surfactant behaviours depend on the chemical structure of the compound. Shown here is the impact of surfactant-containing culture in emulsion formation.

Appendix A6

Appendix A6: Additional information for Chapter 6 (Preliminarily Studies)

A6.1: Materials and methods for generating random mutagenesis

Table A6.1: List of strains used in this research

Strains	Description	Source
Soil isolates (1-58, and controls 1-20)	Strains isolated from Botanic Garden soil	this work
Strain 1.1	ISphoA/hah transposon derivative	
Strain 1.2	ISphoA/hah transposon derivative	
Strain 1.3	ISphoA/hah transposon derivative	
Strain 1.4	ISphoA/hah transposon derivative	
Strain 1.5	ISphoA/hah transposon derivative	
Strain 1.6	ISphoA/hah transposon derivative	
Strain 1.7	ISphoA/hah transposon derivative	
Strain 1.8	ISphoA/hah transposon derivative	
Strain 1.9	ISphoA/hah transposon derivative	

Table A6.2: Primers used in this research

Name	Sequence (5′-3’)	Target	Source/Reference
Forward	GGTCTGAGAGGATGATCAGT	16S rDNA	(Widmer *et al.,* 1998)
Reverse	TTAGCTCCACCTCGCGGC	16S rDNA	(Widmer *et al.,* 1998)
Hah-2	AAACGGGAAAGGTTCCGTCCA	ISphoA/hah	(Giddens *et al.,* 2007)

Table A6.3 Plasmid DNA used in this research

Name	Description	Source
pSCR001 (10571 bp)	The plasmid carried transposon element IS-Omega-Km/hah	Giddens *et al.,* 2007

A6.1.1: Producing surfactant-deficient *Pseudomonas* mutants

The plasmid DNA pSCR001 (Gidden *et al.,* 2007) carrying transposition element IS-Ω-Km/hah was introduced into wild type strain one by electroporation. The mutants produced were selected using KB* plates supplemented with 50 µg/mL kanamycin.

A6.1.2: Electroporation

A modified version of the protocol reported by Francoid *et al.* (1997) was adopted for electroporation. In order to prepare electro-competent pseudomonad, 1mL of the overnight culture was spun and re-suspended in 1mL of ice-cold 10% (v/v) glycerol / 1mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) solution. Cells were washed twice with the glycerol-HEPES solution before re-suspending in 100µL of glycerol / HEPES. 100µL were mixed with 5µL of plasmid before transference to pre-chilled 1mm cap electroporation cuvettes (Flowgen, UK). Electroporation was performed using electroporator 2510 (Eppendorf, UK) within the following conditions: 200Ω, 1.75 kV and 25 mF.

1mL of KB* medium was introduced into the cuvette immediately after electroporation and before transferring the contents into a 1.5mL tube (Eppendorf, UK). The tube was incubated at 28°C and shaken at 200 rpm for 90 minutes. The contents were then transferred into a 6mL microcosm supplemented with 50ug/mL

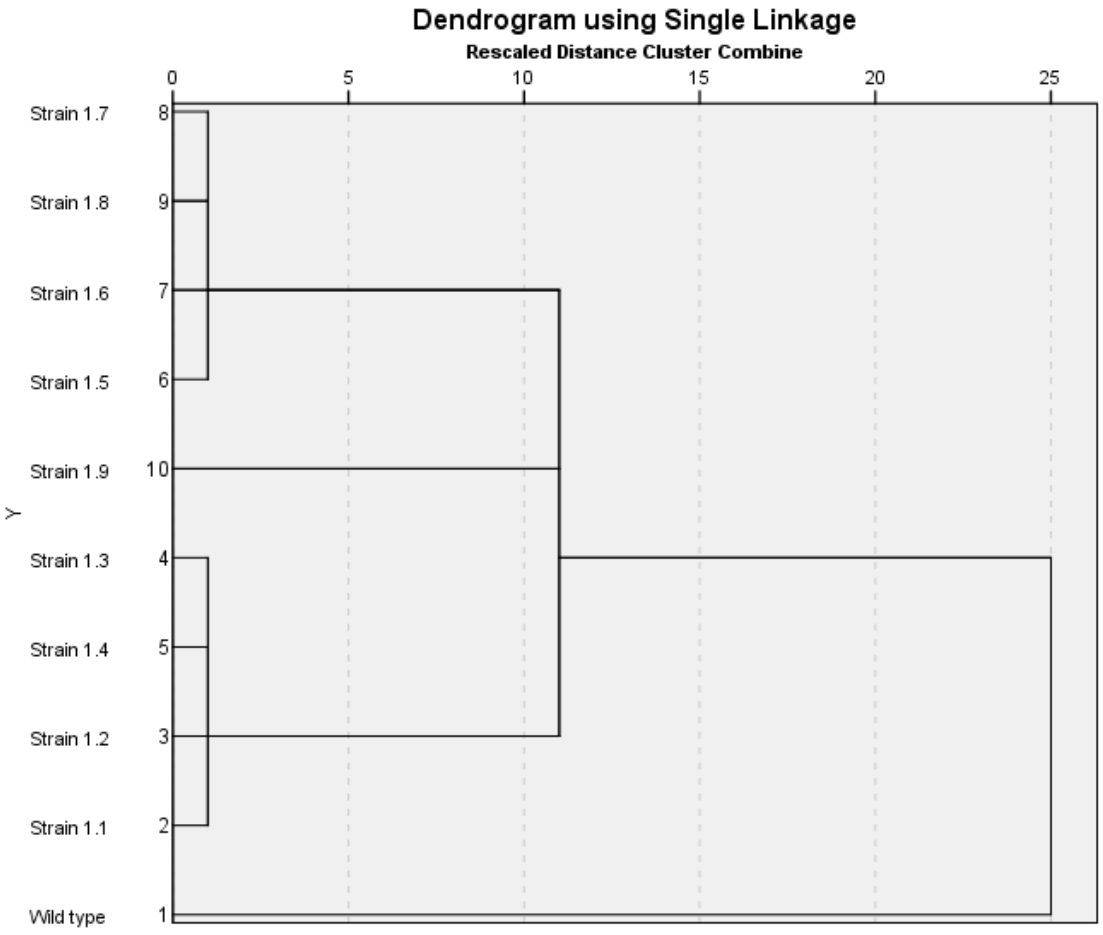
of kanamycin and incubated at 28°C for 24 hours. A library was created by spinning the microcosms and re-suspending them in 1mL of KB* before storing at -80°C.

A6.1.3: Screening mutant library

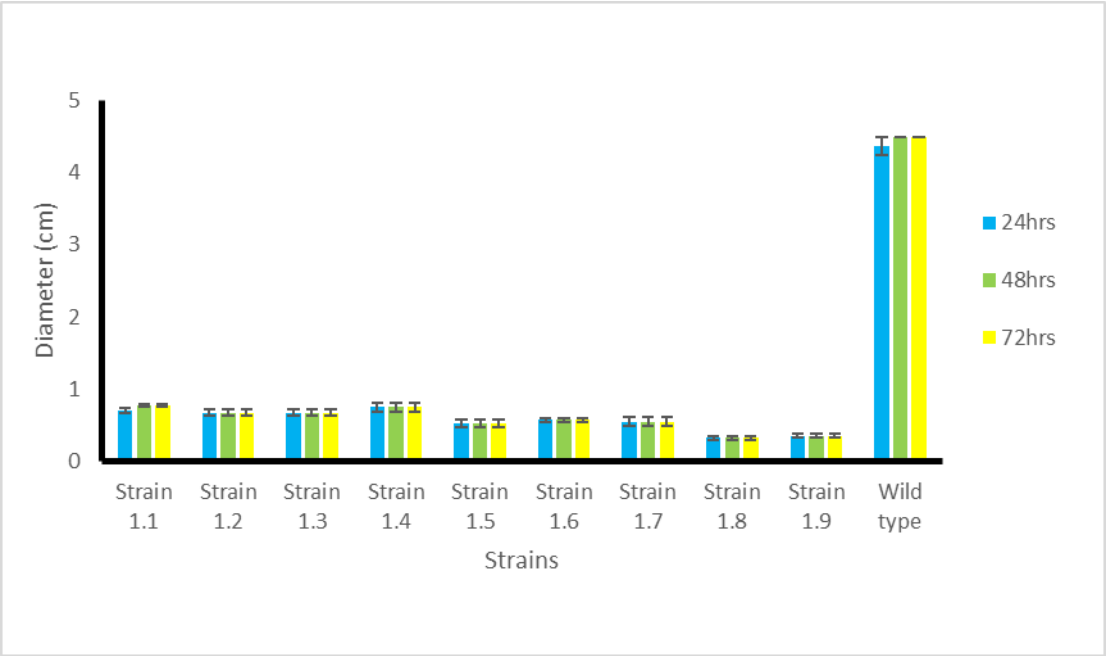
The library generated from the electrophoresis was spread on KB* plates supplemented with 50 µg/mL kanamycin. The colonies obtained were screened for non-surfactant expression using the drop-collapse assay as described in section **2.4**. Glycerol stock of negative strains was prepared and stored at -80°C for further analysis.

A6.1.4 Identification of insert

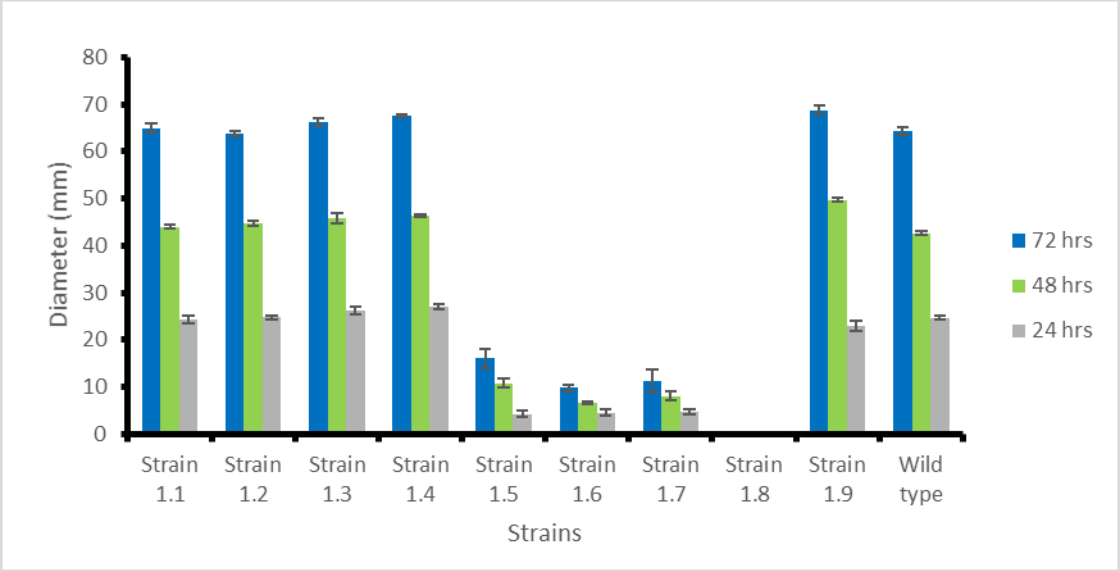
The IS-Ω-Km/hah transposition location in the surfactant-deficient strains was located using cloning techniques. The genomic DNA of the surfactant-deficient strains obtained by electroporation was digested using Kpn1 restriction enzyme. The digested DNA was first cleaned using PCR and Gel purification kits (Bioline, UK) before ligation with DNA ligase enzymes (Bio-labs, UK). The ligated DNA was immediately transformed into competent *E. coli* DH5α and was selected on LB plates supplemented with 50 µg/mL kanamycin. Initial Plasmid DNA extraction and gel electrophoreses confirmed the plasmid clone. The plasmid DNA was diluted and submitted for DNA sequencing.



Appendix A6.2: Diversity among mutants. The data generated from the phenotypic characterisation of the nine mutants and wild type were processed using Hierarchical Cluster Analysis (HCA). Shown here is a dendrogram in which mutant strains show considerable diversity within the collection.



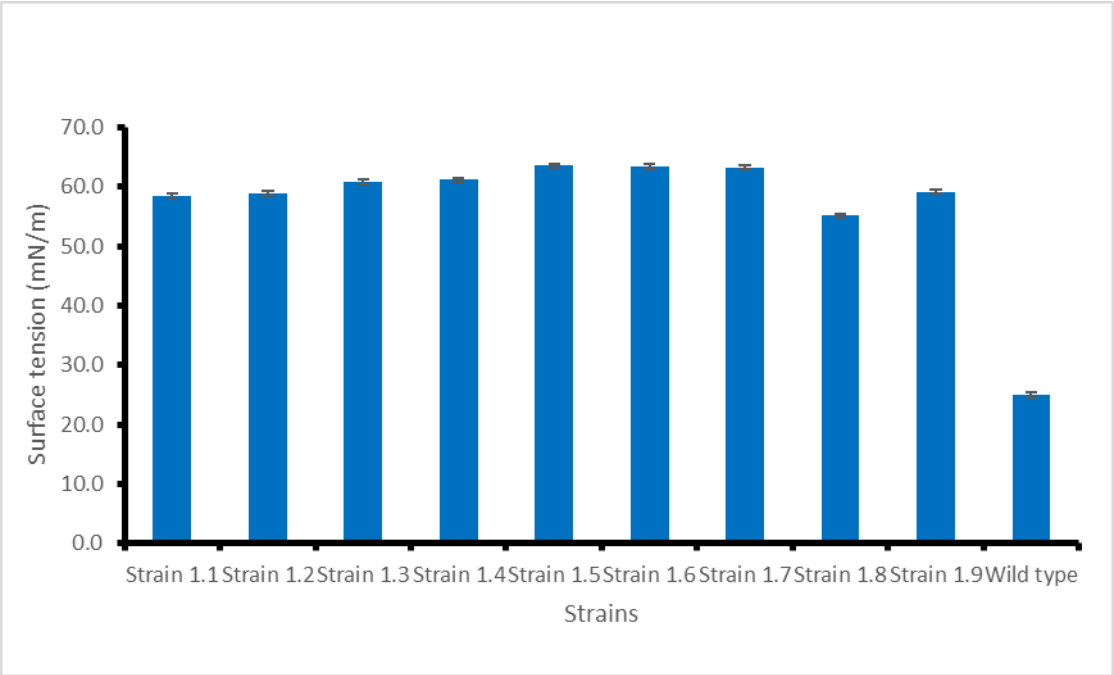
***Appendix A6.3: Loss of swarming character*.** Swarming motility was tested by drop-inoculation of semi-solid KB* plates with mean ± SE (n = 3) measurements shown. There is a significant difference between the wild type and the mutants after 24, 48 and 72 hours respectively ($P < 0.0001$). See Table A6.4 for *P*-values.



Appendix A6.4: Loss of swimming character. Swimming motility was tested by stab-inoculation of 0.3% KB* plates with mean ± SE (n = 3) measurements shown. There is a significant difference between the wild type and mutants after 24, 48 and 72 hours respectively ($P < 0.0001$). See Table A6.4 for *P*-values.

Table A6.4: Motility assays ANOVA P- values

Assays	time (hrs)	DF	F-test	P-value
Swarming	24	9	424.618	0.000
	48	9	871.457	0.000
	72	9	871.457	0.000
Swimming	24	9	251.082	0.000
	48	9	914.227	0.000
	72	9	552.848	0.000



***Appendix A6.5: Assessing mutant strains surface tension reducing ability*.** The liquid surface tension-reducing ability (LSTRA) of mutants was assessed using 18 hour cell-free KB* cultures (n = 4) by quantitative tensiometry. Shown in the above figure are the mean (± SE) surface tensions of the nine mutants plus the wild type. The mutants lost their ability to reduce the surface tension of KB* culture media. A significant difference existed between the mutants and the wild type (ANOVA $F_{9, 20} = 884.464$; $P < 0.0001$).

Appendix A7

Publications related to this work

The experimental work described in this thesis has been published. A copy is included after this appendix.

Conferences

Kamaluddeen Kabir, Yusuf Deeni, Simona Hapca, Corinna Immoor, Sonja Kopanja & Andrew Spiers (2016) Investigating behavioural differences amongst high-performing Pseudomonas spp. surfactants, *Society of Microbiology conference held in Liverpool,* UNITED KINGDOM.

K. Kabir, Y. Deeni, S. Hapca, C. Immoor, S. Kopanja & A. Spiers (2016) Investigating behavioural differences amongst high-performing Pseudomonas spp. surfactants. VAAM Conference, Jena, GERMANY.

Published book Chapter

J. Fechntner, S. Cameron, Y.Y. Deeni, S.M. Hapca, **K. Kabir**, I.U. Mohammed & A.J. Spiers (2017). Limitations of biosurfactant strength produced by bacteria. In: Biosurfactants: Occurrences, Applications and Research. C.R. Upton (Ed.). Nova Publishers.

In: Biosurfactants: Occurrences, Applications and Research ISBN: 978-1-53610-706-7

Editor: Charles R. Upton, pp. 125-148.

Chapter 5

LIMITATION OF BIOSURFACTANT STRENGTH PRODUCED BY BACTERIA

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Abstract

Biosurfactants which reduce the surface or interfacial tension of liquids and act as emulsifiers, foaming and dispersing agents with low toxicity, are finding increasing applications in biotechnology and driving the search for novel compounds for further exploitation. Potential biosurfactants sourced from bacteria are often selected first by qualitative assessment of surface tension using simple assays such as the drop collapse technique or by quantitative tensiometry measuring air-liquid interfacial tension of cell-free culture supernatants or purified samples, and subsequently tested for appropriate physical-chemical behaviours using a range of application-specific assays. Highly active or strong biosurfactants have been reported to reduce the surface tension of water to approximately 22 – 25 $mN.m^{-1}$, and show a range of behaviours determined by the choice of conditions (temperature, pH, salt concentration, etc.) used to test particular aqueous-hydrophobic (oil) mixtures. However, recent analyses of biosurfactant strengths using a predictive statistical approach (Individual distribution identification) have shown that it is unlikely that new compounds will be identified able to significantly reduce aqueous surface tensions below 24 $mN.m^{-1}$. The mechanistic basis of this limit requires an explanation of why stronger compounds are not produced by bacteria, with a limitation of self-harm to producing cells probably the most likely biophysical explanation. However, behavioural analyses using a combination of emulsion, foam stability and oil-dispersion assays indicates high chemical diversity exists amongst biosurfactants exhibiting the strongest levels of activity (24 – 28 $mN.m^{-1}$), suggesting that bacteria are still likely to provide a rich source of potentially novel compounds for use in biotechnology.

Introduction

Biosurfactants produced by bacteria have a range of roles in different environments as well as increasingly-important uses in biotechnology (for a selection of reviews, see [7, 21, 32, 39, 40, 50, 53, 55, 56]). Microbial biosurfactants can be structurally classified into four major classes (glycolipids, lipopeptides/lipoamino acids, polymers including proteins and polysaccharides, and oil/membranes including lipids and fatty acids) (cited in [39]). Both biosurfactant-producing bacteria and the surface-active compounds they express are most usually identified through surveys of bacterial collections or isolates from particular environments, using simple qualitative assays such as the drop collapse technique where the shape of a drop of culture on a glass or plastic surface is assessed. Strains found to be positive for biosurfactant-production are then characterised further, often to the extent of semi-purifying and testing culture supernatants, and sometimes as far as determining the chemical structure of purified compounds. In parallel, the surface activity of biosurfactants is often determined quantitatively by tensiometry measuring the air-liquid interfacial tension (more commonly referred to as the liquid surface tension), and cultures or semi-purified material then tested using assays specific for particular biotechnological applications of interest. Here we also refer to compounds showing high surface activity as 'strong' biosurfactants, and bacteria producing these show significant liquid surface tension reducing abilities [16].

However, the process of identifying novel biosurfactants for biotechnology necessarily starts with a large collection of bacterial strains which are screened with simple qualitative assays to identify biosurfactant-producers, and then by increasingly more specific, time-consuming and expensive quantitative assays, to identify a small number of candidates for pilot-scale testing in which the commercial value of novel biosurfactants can be assessed.

The first biosurfactant to be isolated from bacteria was a cyclic lipopeptide referred to as surfactin, expressed by *Bacillus subtilis* and capable of reducing the liquid surface tension of water from 72 $mN.m^{-1}$ to 27 $mN.m^{-1}$ [6, 23, 24] (reviewed by [46]). Since then, a range of biosurfactants produced by bacteria, mainly by *Bacillus* and *Pseudomonas* spp., have been reported to reduce liquid surface tensions even more down to 22 – 25 $mN.m^{-1}$ (e.g. [11, 16, 17, 22, 26, 27, 28, 35, 36, 38, 42, 48, 57, 60, 67]). However, it is not clear whether substantially stronger biosurfactants are being found in new surveys, as it is hard to compare work carried out over the past 30 – 40 years due to the difficulty of accessing such a large body of literature (see [39] for an illustration of the increase in biosurfactant papers in this period). Furthermore, biosurfactant activities are more generally compared within studies, whilst biology or biotechnology–focussed reviews are not generally representative and are rapidly out-dated. For example, a limit of approximately 29 $mN.m^{-1}$ was suggested in a 2012 review [32] based on an earlier 1997 article listing forty-six research papers [14].

We have addressed this question by taking a statistical approach based on Individual Distribution Identification (IDI) to determine whether a limit to biosurfactant strengths exists, based on our own surveys of collections of bacteria and of published reports, and if so, how close have surveys got to this limit (it is important to note that the limit discussed here is for all bacterial biosurfactants, and is not the same as the limit a particular biosurfactant

approaches as the concentration closes on the critical micelle concentration [Holmberg et al., 2002]). We are also interested to determine the extent of behavioural diversity amongst the strongest group of biosurfactants in order to determine whether one chemical-structural class of biosurfactants is stronger than all other classes, and because a variety of strong biosurfactants with a range of behaviours are clearly required for future biotechnological applications. To illustrate this point, we consider the biosurfactant strengths that might be found in a hypothetical collection of phylogenetically diverse bacteria expressing a range of different biosurfactants (**Figure 1**). Clearly, there must be a limit to the liquid surface tension of culture media that could be used to grow bacteria and produce biosurfactants (the liquid surface tension of aqueous solutions can be significantly reduced by the addition of solvents and solutes, but often at concentrations that would prevent bacterial growth; we have used media with liquid surface tensions of 41 $mN.m^{-1}$ (Lauria broth), 47 – 53 $mN.m^{-1}$ (King's B) and 60 $mN.m^{-1}$ (Minimal M9 Glucose) [16, 35]). Below the liquid surface tension of the culture media we envision a series of downward 'steps' corresponding to the expression of a particular biosurfactant or a group of structurally closely-related homologues (i.e. a class or sub-class of biosurfactants). In this hypothetical case, the width of individual step treads would reflect the number of strains producing a particular biosurfactant, class of biosurfactants, or a mix of different biosurfactants; the first might result from a group of phylogenetically closely-related strains, the second from more distantly-related strains in which sequence diversification has resulted in minor modifications of the ancestral biosurfactant, and the third resulting from strains expressing un-related biosurfactants having the same surface activities. This stepped-pattern also recognises the fact that many compounds expressed by bacteria may have weak surface activities yet are insufficiently strong to be considered a biosurfactant (there is no generally agreed level for surface activity below which a compound is recognised as a biosurfactant, but 30 – 40 $mN.m^{-1}$ seems to be a reasonable threshold, e.g. [40]). We also note that in aging bacterial cultures liquid surface tensions can be significantly increased, suggesting that some bacteria produce 'anti-surfactants' [16, 35].

– FIGURE 1 TO GO NEAR HERE –

However, in several surveys of bacterial collections [16, 35] and in on-going work (**Figure 2**), we have found very smooth distributions of biosurfactant strengths. This may be a result of within–strain (replicate) variation in measurements, interactions between biosurfactants and other compounds differentially expressed by strains, as well as a more relevant and interesting diversity within and between biosurfactant classes.

– FIGURE 2 TO GO NEAR HERE –

Estimating a limit to biosurfactant strength

Biosurfactant strengths can also be examined using a statistical approach which aims to identify a probability distribution model that provides the best fit to the observed data [10]. Different statistical packages such as Minitab (Minitab Inc.), Matlab (The Mathworks Inc.),

or SAS (SAS Institute Inc.) have implemented tools for probability distribution fitting tools. In Minitab (v.16.) this is done via the Individual Distribution Identification (IDI) tool which has the capability to fit sixteen individual distributions including the normal, log-normal, exponential, two or three parameter gamma, and two or three parameter Weibull distribution as well as Johnstone transformation for normal distribution fit [37]. As part of this tool the Anderson-Darling (AD) goodness of fit test is used for best distribution model selection based on the test statistic and the corresponding p-value (a god fit is reflected in a low AD test statistic and a high p-value). A lower limit to the expected biosurfactant strength can be then calculated from the model parameter estimates.

We have used this approach to estimate the the lower limit of biosurfactant strengths from a number of surveys of bacteria in which we determined liquid surface tensions from cell-free culture supernatants or obtained similar data from research publications. We originally screened a comprehensive collection of environmental pseudomonads we had acquired during other research, including many plant pathogens, plant and soil-associated strains, as well as a set of pseudomonads directly isolated from sandy loam soil [16], and have continued with this type of analysis with a more diverse collection of pseudomonads or *Pseudomonas*–like bacteria isolated from activated sludge from a waste-water treatment system and oil-contaminated soil [35]. Our current research is focussed on a small number of fluorescent pseudomonads recovered from directly below the roots of grass growing in uncontaminated soil. IDI analyses of these data all predict a similar lower limit for bacterial biosurfactant strength (γ_{Min}) of 24 $mN.m^{-1}$ (**Table 1**). This limit is probably not specific to pseudomonads, as an analysis of surface tension strengths published for fifty-nine bacterial strains including eight *Bacillus* spp. results in a similar prediction [35]. Our review of the literature suggests that surface tensions for high-strength bacterial biosurfactants range from 22 – 25 $mN.m^{-1}$ (see [35] and references therein), with the lowest resulting from an un-replicated measurement with no indication of reproducibility [67]. We note that recent publications have not listed any stronger biosurfactants (**Table 2**), and as a result, we remain confident of our prediction of the lower limit for bacterial biosurfactant strength.

– TABLE 1 TO GO NEAR HERE –

– TABLE 2 TO GO NEAR HERE –

Mechanistic basis of the limit

The mechanism or mechanisms limiting the production of stronger biosurfactants currently lacks a detailed explanation, although we suggest that a limitation of self-harm to producing cells is probably the most likely biophysical explanation. All biosurfactants are synthesised in the cytosol though ribosomal or non-ribosomal peptide synthesis [18] and specific biosynthetic enzyme activity, and at each stage, intermediates or the final compounds could have a detrimental biophysical effect on the producing cell.

Lipopeptide biosurfactants, such as fengicin, iturin and surfactin, produced by *Bacillus* spp. and amphicin, syringomycin and viscosin, produced by *Pseudomonas* spp. [54] are synthesised via relatively unusual non-ribosomal peptide synthase (NRPS) enzyme complexes [34]. These enzyme complexes are encoded by gene clusters and are modularly organised, allowing different modules to combine in the production of different biosurfactants. These complexes drive the amino acid addition of the peptide portion of lipopeptide biosurfactants to a fatty acid moiety produced from cellular fatty acid metabolism, in an amino to carboxy-terminus direction with cyclisation in some lipopeptides [34, 54]. Global regulation of these complexes is controlled at a transcriptional level by two-component systems such as ComP/ComA and DegS/DegU in *Bacillus* spp. [33, 66] and similar two-component systems in *Pseudomonas* spp. [25, 41] to control production of these biosurfactants depending on the stage of the cell cycle and environmental conditions.

Glycolipid biosurfactants, such as the rhamnolipids produced by *Pseudomonas* spp. and trehalose lipid by *Rhodococcus* spp., are synthesised by specific enzymes. Lipid components of glycolipids proceed through the classical fatty acid synthesis, where, in the example of rhamnolipids, the β-hydroxydecanoyl-ACP intermediate from the FASII cycle is sequestered by RhlA, and RhlB and RhlC then catalyse the transfer of rhamnose from dTDP-rhamnose to form mono or di-rhmanolipids [3, 51]. RhlAB is both transcriptionally and post-transcriptionally regulated by factors related to quorum sensing and is also involved in swarming and biofilm formation [12, 31, 51]. The dTDP-rhamnose itself is synthesised in bacteria from glucose by RmlABCD and is transcriptionally auto-regulated by RmlA [51].

The chemical nature of biosurfactants makes them inherently amphiphilic with fatty acid moieties which are hydrophobic, have an affinity for other amphipathic molecules (i.e. the major components of biological membranes) through hydrophobic or other interactions, and have demonstrated toxicity to bacterial cells [30, 65]. The physicochemical nature of biosurfactants is the most likely mechanism limiting the production of stronger compounds, creating a selective pressure by which the development of stronger biosurfactants leads to unsustainable self-damage, and is therefore genetically and phenotypically unfavourable.

The major phospholipid and membrane component in bacteria is phosphatidylethanolamine, with some bacterial membranes also containing phosphatidylcholine [61]. Bacteria which produce biosurfactants must therefore have membranes containing these molecules which are not disrupted by the levels of biosurfactants they produce inside and around the cell. Lipopeptide biosurfactants have been shown to lead to the permeabilisation of biological membranes [9], by interacting with phospholipid acyl chains and causing the formation of ion-conducting pores [8, 19]. This disrupts membrane integrity causing leakage and effecting normal cell function [8, 13, 19, 20]. Indeed, many lipopeptides are categorised as antibiotics, with some molecules used as clinical antimicrobials (e.g. daptomycin and polymyxins) [47], because they kill bacterial cells via membrane disruption. Alteration of the cell surface including changes to lipopolysaccharide (LPS) and lipid A leads to reduced sensitivity to polymyxin antimicrobials [43], suggesting that biosurfactants directly damage biological membranes, with stronger molecules potentially disrupting membrane function further.

Glyocolipids, particularly the rhamnolipids, have been more widely studied to understand the effects they have on cells and cell membranes. Studies in a variety of different bacteria, including *Pseudomonas* and *Bacillus* spp., and model membranes, have demonstrated that the rhamnolipids alter the biophysical properties of membranes leading to destabilisation and permeabilisation [1, 2, 58, 59, 62, 63]. More detailed studies on the effects of rhamnolipids on *Pseudomonas aeruginosa* have demonstrated a marked decrease in the levels of major outer membrane proteins OprDFJM, leading to a thinner and more compact structure [64] that resists the permeabilisation and destabilisation of the membrane. A reduction in these outer membrane proteins would lead to less regulation molecules and ions moving across the membrane and reduce structural integrity. Exposure to rhamnolipid also leads to a decrease in LPS by solubilisation and by complexing Mg^{2+} [4, 64]. LPS is a major component of Gram-negative bacteria outer membranes, contributing to the structural integrity of the cell and stabilising the membrane. Trehalose lipid has also been shown to disrupt membranes through interactions with phosphatidylethanolamine and phosphatidycholine, altering lipid fluidity which is an essential component of bacterial membrane function [5, 44].

Together these observations suggests that stronger biosurfactants may have an increased ability to disrupt membranes and cause self-harm in the bacteria producing them, leading to a strong evolutionary selection disadvantage which has limited their emergence. However, there are other mechanisms which may limit the production of stronger biosurfactants. One potential mechanism is the increased ability of stronger biosurfactants to solubilise or disrupt proteins or other macromolecules inside cells which could interrupt essential metabolic pathways or secretion systems; these may be indirectly or directly linked to biosurfactant production, disruption of secretion mechanisms might also lead to the accumulation of intermediates or products which may be toxic. The production of stronger biosurfactants might also be restricted by community interactions of producer cells in biofilms, with stronger compounds disrupting cell signalling or reducing surface tension to a point where biofilms are weak or poorly formed.

Evidence for chemical diversity amongst the strongest biosurfactants

We are also interested in determining the extent of chemical diversity amongst the strongest group of bacterial biosurfactants and are focussing on a group of fluorescent pseudomonads which produce liquid surface tensions in the range of 24 – 26 $mN.m^{-1}$ in cell-free culture supernatants. Although this group may be phylogenetically similar, the pseudomonads are known to produce several different classes of biosurfactants, including the cyclic lipo-peptides and rhamnolipids [39, 50]. Ultimately the best way to assess biosurfactant diversity would be to purify compounds and determine chemical structures, but this approach even for a single example is both expensive and time-consuming, and is unpractical for a large collection of samples. An alternative approach would be to obtain whole genome draft sequences for each bacterial strain and to identify candidate biosurfactant synthesis genes by DNA and protein sequenced-based homologies to infer biosurfactant structures at least to the

class level (this is no longer unpractical even for large collections). However, additional investigation would be needed to confirm that the candidate genes were involved in biosurfactant synthesis, and then structural analysis required to confirm the type of biosurfactant produced.

We have therefor taken a different approach to assess the diversity of biosurfactants produced by this group of pseudomonads, and have employed a number of simple foam, emulsion and oil-displacement assays to compare behaviours using cell-free culture supernatants or semi-purified biosurfactant samples (for a list of assays that can be used to characterise biosurfactants see [39, 68]). Clearly other compounds present in the supernatants or samples might interact with the biosurfactant to alter behaviours in some assays, though this would be minimised in analyses of closely-related bacteria. Although we have examined the data from individual assays directly (**Figure 3**), we have found that it is more informative to use a multivariate exploratory statistical method such as hierarchical cluster analysis (HCA) to look at similarities between isolates [15].

– FIGURE 3 TO GO NEAR HERE –

The HCA output is in the form of rectilinear cladograms or star-burst–like constellation diagrams from which groups or clusters of similar isolates can be drawn. Furthermore, it is possible to investigate similarities between assay data by two-way clustering, and preliminary analysis of a test set of strains could be used to determine which assays best differentiate between strains before the entire collection is assessed. In our behavioural analysis of pseudomonad biosurfactants, we have used diesel, mineral oil and vegetable oil–based displacement assays in which the oil film overlays an aqueous layer of water (pH 6.0) or Tris-buffer (pH 8.0). The resulting constellation diagram separates the twenty-five biosurfactant producing strains and five negative controls into four large clusters, demonstrating that significant biosurfactant chemical and behavioural diversity exists within this collection (**Figure 4**), whilst the two-way clustering information indicates that the three oils differentiates the strains more than the pH of the aqueous layer. If we were to go on to determine the structures of five biosurfactants produced by these pseudomonads, we would choose two strain pairs from the two most distant clusters to maximise the chance of finding different structural classes and different types within classes, e.g. Strains 2 and 3 from the top-right cluster and 6 and 13 from the bottom left cluster shown in **Figure 4**. Alternatively, our choice of candidates for further analysis might also take into consideration particular assay results which might be more relevant for proposed applications, e.g. Strains 7 and 24 show particularly high levels of activity in the oil displacement assay shown in Figure 3 and these might be more interesting for further analysis. It should be noted that choosing within-cluster pairs is risky as the biosurfactants they produce may be identical, and the minor differences shown in the constellation diagram might be due to the presence of other compounds differentially produced by the strains which interfere with biosurfactant behaviours.

– FIGURE 4 TO GO NEAR HERE –

In this analysis of strong biosurfactant-producing pseudomonads and other work [35, 52], we have also been interested in differentiating strains based on phenotype using a HCA approach. A range of simple growth, biochemical and plate-based assays can be used to demonstrate that a collection is diverse and contains few biological replicates (i.e. a strain isolated more than once from the same sample or from two sampling sites close together), and often is more informative than analytical profile index (API)–like testing or 16S rDNA sequence analysis. This data can also be used to select candidate strains for further analysis as discussed above, as well as provide useful information about culture requirements which might be important when considering larger scale production to isolate biosurfactants for testing or analysis.

CONCLUSION

Biosurfactants continue to be of interest in biotechnology, and new compounds are often selected from a relatively small set of biosurfactant-producing bacterial isolates where a key characteristic is surface activity or strength. Using a statistical approach, we have recently demonstrated that there is a limit to biosurfactant strength of approximately 24 $mN.m^{-1}$. We suggest that stronger compounds are not produced by bacteria because of the need to reduce self-harm to the producing cells, though this mechanistic explanation requires experimental confirmation. However, despite a limit to biosurfactant strength, it is clear that even within small collections of bacteria expressing strong biosurfactants that sufficient chemical variation exists to satisfy the need for biosurfactants with different behavioural characteristics suitable for a range of biotechnological applications.

Acknowledgments

We acknowledge the support the Abertay Graduate School has provided to our PhD students, Kamaluddeen Kabir and Ibrahim Mohammed. Kamaluddeen also received funding from TetFund and Umaru Musa Yar'adua University, Nigeria, as well as from the Society for Microbiology and Society for Applied Microbiology. Ibrahim was funded by the Nigerian Petroleum Technology Development Fund. Joanna Fechtner was a MSc(Hons) project student at Abertay. Andrew Spiers is also a member of the Scottish Alliance for Geoscience Environment and Society (SAGES).

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Figure Legends

Figure 1. **Bacteria produce a range of biosurfactants of varying strengths.** Shown here is a hypothetical distribution of the liquid surface tension reducing abilities of a collection of bacteria producing a range of surface active compounds including biosurfactants. Strains shown ranked from strongest (left) to weakest (right) along the *x*-axis with an arbitrary measure of liquid surface tension on the *y*-axis. The action of a biosurfactant in an aqueous solution such as culture medium is to lower the liquid surface tension (A) (strong biosurfactants show the greatest activity and lower liquid surface tension). Some surface active compounds may show sufficient strength to be considered biosurfactants (black arrows), whilst others may only have a weak effect (grey zone) or act to increase liquid surface tension (grey arrow). However, in any survey of bacteria, there will be one expressing a biosurfactant with the strongest activity (B). It is possible that stronger biosurfactants might be identified in further work, but there must also be a physical limit to the extent to which liquid surface tensions can be reduced and still allow bacterial growth (C). The diversity of strains and biosurfactants will decrease with increasing strength, with relatively few strains producing strong biosurfactants, and potentially one type or group of biosurfactants having the strongest activity.

Figure 2. **Significant variation in biosurfactant strengths are seen in collections of biosurfactant-producing bacteria.** Shown here are the results of quantitative tensiometry of cell-free King's B culture supernatants of a collection of *Pseudomonas* spp. strains ranked in order of surface activity (strength). The dashed line (A) indicates the liquid surface tension of sterile King's B medium, and the solid bar (B) indicates a homogeneous set of twenty-five strong biosurfactant-producing bacteria (identified by *post hoc* Tukey-Kramer HSD, α = 0.05) which are examined in further work (including Table 1 and Fig. 3 here). Means and standard errors are shown. Data are from K. Kabir & A.J. Spiers (unpublished observations) and will be published in full elsewhere.

Figure 3. **Behavioural diversity can be assessed by simple quantitative assays.** Shown here are the results of an oil displacement assay using cell-free culture supernatants and mineral oil overlaid on pure water. Twenty-five *Pseudomonas* spp. strains produce biosurfactants and show considerable variation in the displacement of oil films, whilst the five negative control strains (Ctrls) show negligible activity. Means and standard errors are shown. Data are from K. Kabir & A.J. Spiers (unpublished observations) and will be published in full elsewhere.

Figure 4. **Significant behavioural diversity exists within the group of biosurfactants with the highest activity.** Behavioural assays can be used to assess the diversity of biosurfactants within a collection in order to identify candidates for further characterisation or testing for a biotechnological application. Shown here is a constellation diagram showing similarities between thirty *Pseudomonas* spp. strains, of which twenty-five express biosurfactants (grey

circles) and five controls which do not (white circles). Similar strains are grouped together, with the four main groups indicated by dashed circles; dissimilar strains are those found the furthest apart on the diagram. This diagram was produced by a hierarchical cluster analysis (HCA) of oil displacement assays testing diesel, mineral and vegetable oil overlaid on water (pH 6.0) or Tris-buffer (pH 8.0) water. The diagram is rooted arbitrarily mid-way along the longest branch (circled dot). In the top left the clustering of the six assays is shown, indicating that behaviours were more similar within than between oils. Data are from K. Kabir & A.J. Spiers (unpublished observations) and will be published in full elsewhere.

Tables –

Table 1. Predicted limit for liquid surface tensions produced by bacterial biosurfactants

Origin of bacteria	Best fitting 3-parameter distribution	*N*	*P*	AD	Predicted limit ($mN.m^{-1}$)	Reference
Contaminated soil and activated sludge	Log-logistic	50	0.294	0.497	24.24	[35]
Soil	Gamma	38	0.233	0.688	24.16	[16]
Soil	Log-normal	25	0.784	0.237	24.74	*
Random sampling of published reports	Weibull	59	0.386	0.238	24.23	[35]

N, Number of bacterial strains samples; *P*, p-value (a large value is required); AD, Anderson-Darling test statistic. Individual distribution identification (IDI) was used to fit theoretical probability distributions to liquid surface tension data, and the minimum liquid surface tension predicted from the threshold parameters. Only the best-fitting distributions are listed. * Data and analyses are from K. Kabir & A.J. Spiers (unpublished observations) and will be published in full elsewhere.

Table 2. Recent reports of bacterial biosurfactant strengths (2015 – 2016)

Bacteria	Liquid surface tension ($mN.m^{-1}$)	Reference
Bacillus spp. strains	28.6 – 60.4	[48]
Bacillus licheniformis R2	28	[22]
Bacillus subtilis M15-10-1	~30	[17]
Corynebacterium xerosis NS5	31.4	[11]
Lactic acid bacteria, various spp.	~55 – 75	[49]
Pseudomonas aeruginosa DSVP2	~30	[60]
Rouxiella sp. DSM 100043	28	[27]
Tsukamurella pseudospumae DSM44118	28.7	[26]
Oil-degrading marine bacteria, various spp.	28.2 – 52.7	[36]
Oil-contaminated soil, various spp.	< 40	[29]
Petrochemical-contaminated soil, various spp.	~50 – 60	[45]

Papers reporting the liquid surface tension of biosurfactants expressed by bacteria were selected using PubMed (on the 28 August 2016) with the key words ‘bacteria’ and ‘surfactant’ and with a publication date range of September 2015 – August 2016. Approximate strengths are those determined from liquid surface tension figures.

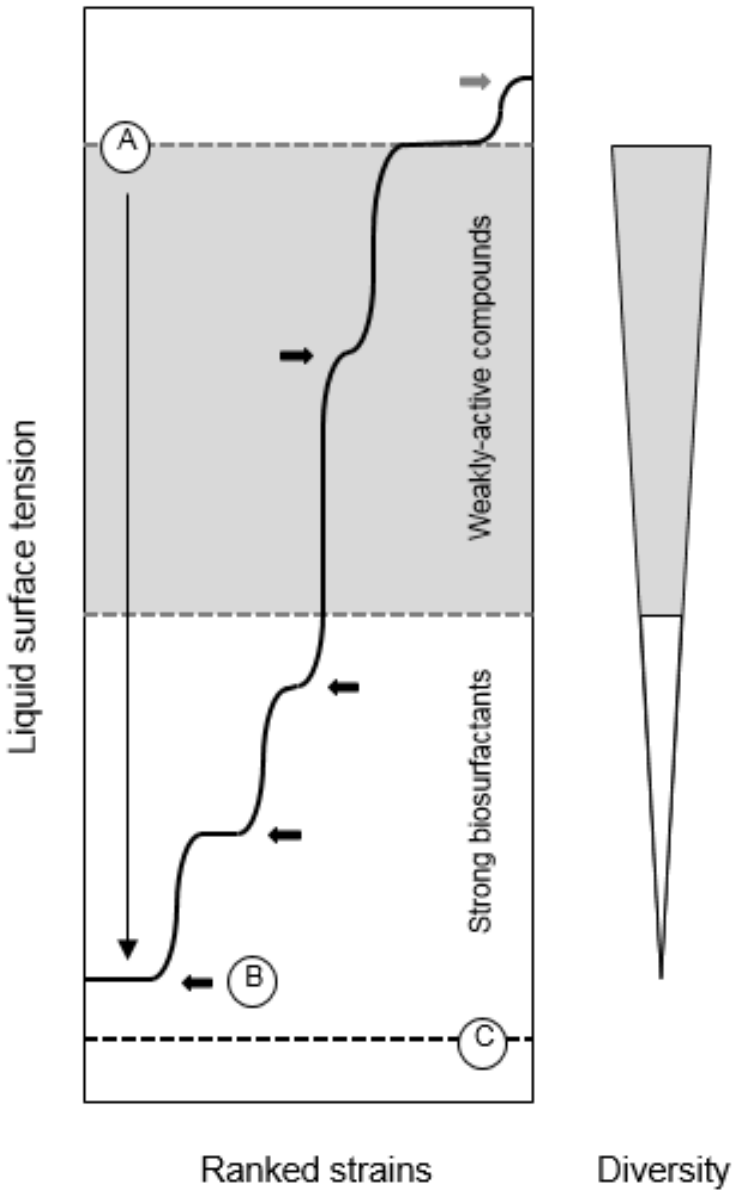
A
B
C
Weakly-active compounds
Strong biosurfactants
Liquid surface tension
Ranked strains
Diversity

Figure 1

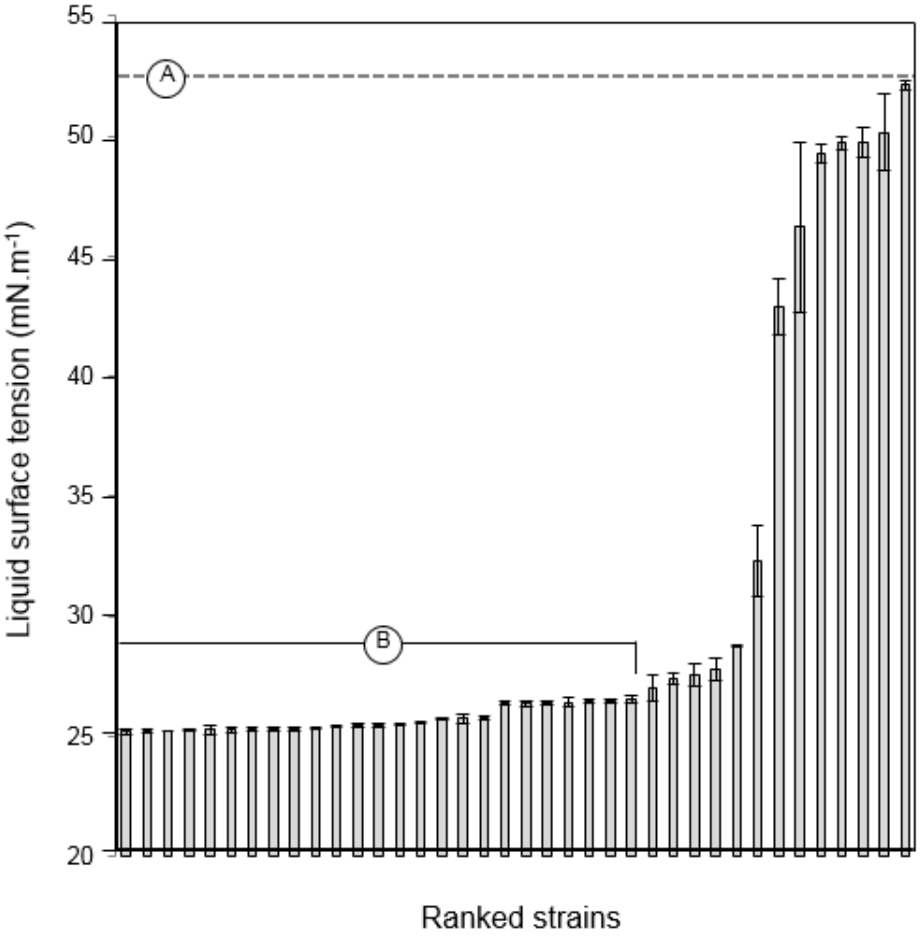
55
50
45
40
35
30
25
20
A
B
Liquid surface tension (mN.m-1)
Ranked strains

Figure 2

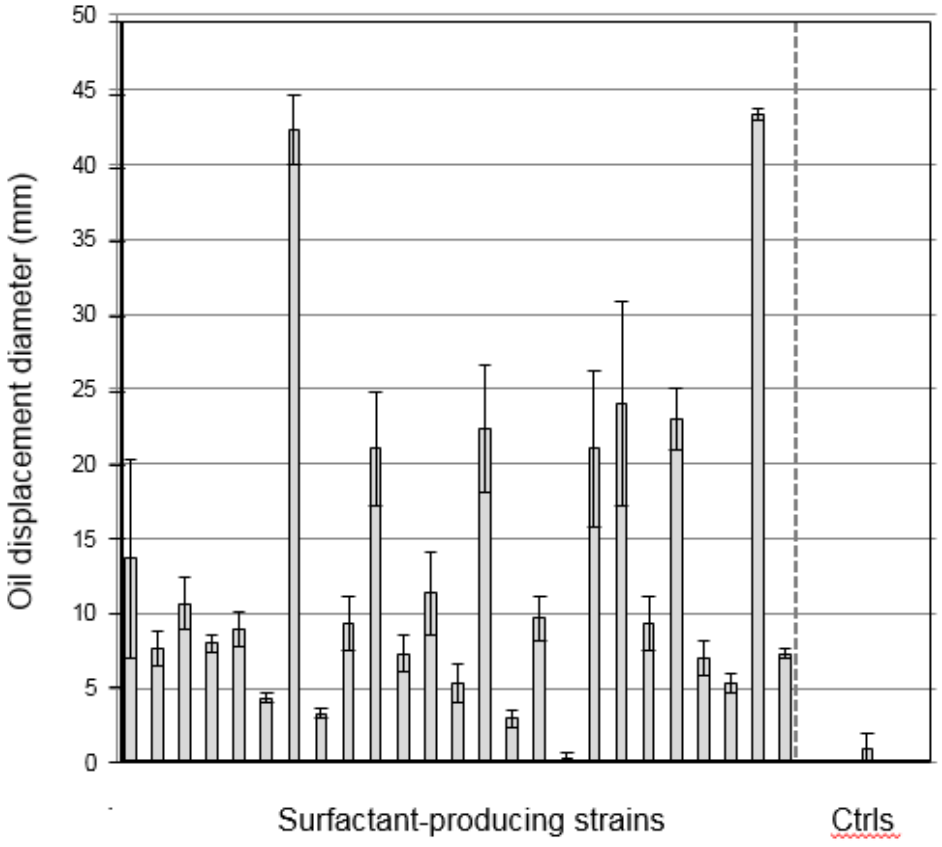
Oil displacement diameter (mm)
50
45
40
35
30
25
20
15
10
5
0
Surfactant-producing strains
Ctrls

Figure 3

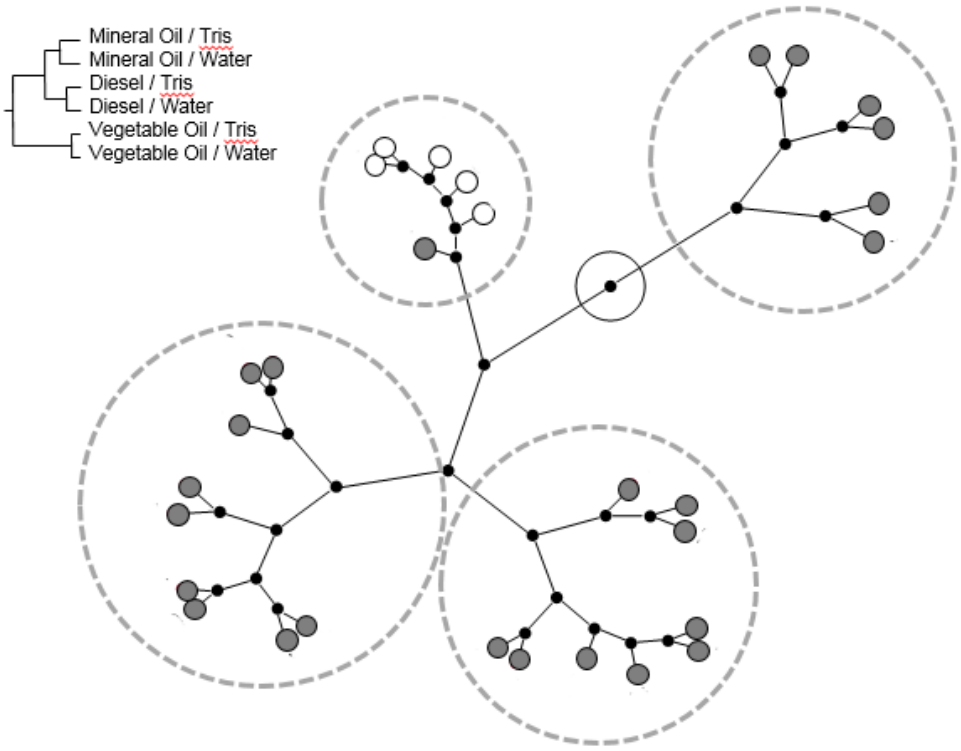
Mineral Oil / Tris
Mineral Oil / Water
Diesel / Tris
Diesel / Water
Vegetable Oil / Tris
Vegetable Oil / Water

Figure 3